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Metastasis of breast cancers contributes heavily to the severity and mortality of this disease. There is substantial evidence that integrins and small GTPases contribute to the metastatic properties of breast cancer cells enabling them to invade and migrate to distant sites throughout the body. This study provides evidence that Rac GTPase is required for both integrin and growth factor-mediated motility. Although Rac can activate JNK, not MAP kinase, we found it can interact with the Ras/MAP kinase pathway at the level of Raf kinase to regulate cell migration. One of the identified targets of Rac-GTP in cells are the p21-activated kinases (PAKs). PAKs can mediate many of the cytoskeletal changes induced by Rac and Cdc42. We show that cell adhesion can activate PAK through an integrin-dependent manner and PAK mediates cell spread and motility on extracellular matrix.

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INTRODUCTION

Metastasis of breast cancers contributes heavily to the severity and mortality of this disease. There is substantial evidence that integrins and small GTPases contribute to the metastatic properties of breast cancer cells enabling them to invade and migrate to distant sites throughout the body. This study provides evidence that Rac GTPase is required for both integrin and growth factor-mediated motility. Although Rac can activate JNK, not MAP kinase, we found it can interact with the Ras/MAP kinase pathway at the level of Raf kinase to regulate cell migration. One of the identified targets of Rac-GTP in cells are the p21-activated kinases (PAKs). PAKs can mediate many of the cytoskeletal changes induced by Rac and Cdc42. We show that cell adhesion can activate PAK through an integrin-dependent manner and PAK mediates cell spread and motility on extracellular matrix.

RESULTS

Expression of Tiam1 induces cell migration

Breast tumor cells like all transformed cells tend to be anchorage independent and thus demonstrate constitutively activated signaling molecules. Therefore, to begin to understand which of these signaling molecules might contribute to the metastatic capacity of breast cancer, I examined a non-malignant cell lines that could be induced to migrate by expression of activated signaling molecules. COS-7 cells were transfected with Tiam1, a Rac activator,, and then allowed to migrate on a collagen substrate. Tiam1-expressing cells have a 3-4 fold increase in cell motility on collagen. Cotransfection with a dominant negative Rac T17N blocked Tiam1 enhancement of cell motility about 70%. These results suggest that Tiam1 can promote cell migration on collagen in a Rac-dependent manner.

Rac activation is necessary for integrin-mediated cell migration

The Rho-family of Ras-related GTPases is required for assembly of the actin cytoskeleton and associated focal complexes. Rac stimulates the formation of membrane ruffling through actin cytoskeleton reorganization. To further investigate the role of Rac in integrin-mediated cell motility, dominant active and negative Rac was tested. Dominant negative Rac and Cdc 42 blocked integrin-mediated cell migration significantly. Unexpectedly, overexpression of dominant active Rac Q61L did not alter cell migration on collagen. These finding suggest that Rac is necessary but not sufficient for cell migration on collagen.

Rac and Raf synergize to activate MAP kinase and promote cell motility

Integrin ligation leads to the activation of focal adhesion kinase with subsequent activation of Ras/MAP kinase pathway. Recently, MAP kinase was shown to affect cell motility by regulating the activity of myosin light chain kinase (MLCK). Although Rac activates the JNK pathway and has little effect on MAP kinase activity, it appears to play an essential role in Ras transformation. Thus, it is possible that Rac is involved in the regulation of cell motility by MAP kinase. To address this issue, we examined the effects of Rac activation on MAP kinase activation and the

induction of cell motility by Raf kinase. Rac expression together with low level Raf can not only activate MAP kinase, but can also promote cell migration on collagen.

To further study the mechanism by which Rac affects integrin-mediated cell motility, various inhibitors of potential downstream targets were tested. Pretreatment of cells with a MAP kinase kinase (MEK) inhibitor (PD98059), which specifically prevents its ability to promote both threonine and tyrosine phosphorylation of MAP kinase, completely blocks Tiam1-induced cell motility. This suggest MAP kinase is downstream of Rac and necessary for this migration event.

Rac GTPase and PAK are activated by adhesion

To examine the effects of integrin ligation on Rac activity, quiescent NIH3T3 cell were plated on FN-coated dishes. Cells adhere within 5 min and spread over a period of 60 min. During this period, cells extend filopodia and show extensive membrane ruffles. These results suggest that Rac and Cdc42 may be activated during cell adhesion and spreading.

Direct assays of Rac or Cdc42 activation are technically difficult, therefore, we assayed the serine/threonine kinase PAK, which is the direct downstream target of these GTPases. Rac and Cdc42 interact with a number of effector proteins. The best characterized effectors are the PAKs. Activated PAK1 mutants themselves, like Rac, induce the formation of lamellipodia. Plating cells on FN-coated dishes strongly stimulated PAK. Activation was also observed when cells were plated on an antibody to ß1 integrin but not on FN-40 kDa fragment, to which cells adhere via heparin independent of integrin. These results demonstrated that integrin-dependent adhesion specifically activates PAK, by inference, Rac and /or Cdc42.

Ras induces angiogenesis

One of the important features of tumor is its ability to release angiogenic factors and trigger signaling transductions to induce angiogenesis. To examine the role of Rac and PAK in cancer cell metastasis and their ability to induce angiogenesis, we employed a CAM model on chick embryo, since this model had been widely used to study breast tumor growth, metastasis and angiogenesis. We used recombinant adenovirus to delivery signaling molecules onto CAM of chick embryos *in*

vivo. Our results indicated that bFGF and VEGF-mediated angiogenesis is Ras-dependent, and active form of Ras, Ras G12V, induces angiogenesis in an integrin αvß3 and αvß5-dependent manner.

In conclusion, we found Rac and PAK activity is required for integrin or growth factor-mediated cell spread and movement. they co-operate with MAP kinase pathway to regulate actin/myosin reorganization. Using an *in vivo* gene delivery system, we investigated the roles of Ras, Rac and PAK in breast tumor growth and tumor angiogenesis. Considering Rac and PAK signaling appears to be abnormal in breast cancer, this study will increase our understanding of abnormal cellular regulation in breast cancer and may lead to identification of direct therapeutic targets capable of inhibiting of tumor progression and metastasis.

APPENDICES

Research accomplishments

Manuscripts

Klemke R. L. **Leng J.**, Molander, R., Brooks, P., Vuori, K., and Cheresh, D.A. 1998. CAS/Crk coupling serves as a molecular Switch for induction of cell migration. *J. Cell Biol.*, 140: 961-72.

Leo, P., **Leng, J.**, Schwartz, M.A., and Bokoch, G.M. 1998. Activation of Rac and Cdc42 by integrins mediates cell spreading. *Mol. Biol. Cell* 9:1863-71.

Cheresh, D.A., **Leng, J.**, and Klemke, R.L. 1999. Distinct signaling pathways control membrane ruffles and actin/myosin motor activity. *J. Cell Biol.*, 146: 1107-16.

Leng, J., Klemke, R.L., Reddy, A., and Cheresh, D.A. 1999. Potentiation of cell migration by cooperative signals from small GTPase Rac and Raf Kinase, submitted to *J. Biol. Chem.*.

Abstracts

Klemke R. L. **Leng J.**, Molander, R., Brooks, P., Vuori, K., and Cheresh, D.A. 1998. Regulation of cell migration by CAS/Crk coupling. *Keystone symposia: Motility and Meastasis.* 66:002.

Leng J., Reddy A., Klemke R., and Cheresh D.A. 1998. Dual roles for the GTPase, Rac in cell migration. 38th American Society for Cell Biology Annual Meeting. *Supplement to Molecular Biology of the Cell*, 9:1686.

Leng J., Klemke R., Reddy A., and Cheresh D.A. 1998. Dual roles for the GTPase, Rac in cell migration. *Keystone symposia: Oncogene Networks in Signal Transduction.* 186:2129.

Employment opportunities

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Activation of Rac and Cdc42 by Integrins Mediates Cell Spreading

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Adhesion to ECM is required for many cell functions including cytoskeletal organization, migration, and proliferation. We observed that when cells first adhere to extracellular matrix, they spread rapidly by extending filopodia-like projections and lamellipodia. These structures are similar to the Rac- and Cdc42-dependent structures observed in growth factor-stimulated cells. We therefore investigated the involvement of Rac and Cdc42 in adhesion and spreading on the ECM protein fibronectin. We found that integrin-dependent adhesion led to the rapid activation of p21-activated kinase, a downstream effector of Cdc42 and Rac, suggesting that integrins activate at least one of these GTPases. Dominant negative mutants of Rac and Cdc42 inhibit cell spreading in such a way as to suggest that integrins activate Cdc42, which leads to the subsequent activation of Rac; both GTPases then contribute to cell spreading. These results demonstrate that initial integrin-dependent activation of Rac and Cdc42 mediates cell spreading.

INTRODUCTION

Most cell types respond to surfaces coated with ECM proteins by adhering and then spreading out to acquire a flattened morphology. This process of cell adhesion and spreading is mediated by integrins and involves complex dynamic rearrangements of the actin cytoskeleton. These dynamics appear to be coordinated in space and time by intracellular signaling pathways involving tyrosine kinases, protein kinase C, arachidonic acid metabolism, and, in some cases, intracellular calcium (Chun and Jacobson, 1992, 1993; Pelletier *et al.*, 1992; Auer *et al.*, 1993; Vuori and Ruoslahti, 1993; Romer *et al.*, 1994). How specific signaling pathways regulate the cytoskeleton is, however, poorly understood.

Cells spread by putting out extensions that contact the surface, form adhesions, and then exert tension to induce outward movement. This process is reminiscent of the extensions and adhesions induced by the small GTP-binding proteins Rac and Cdc42. These proteins are closely related members of the Ras superfamily of GTPases, which, like other Ras family members, act as guanine nucleotide-regulated switches. Cdc42 mediates formation of long, thin, actin-dependent extensions called filopodia, whereas Rac mediates formation of curtain-like extensions called lamellipodia and ruffles (Ridley *et al.*, 1992; Nobes and Hall, 1995). Both can induce formation of small substrate adhesions called focal complexes.

Rac and Cdc42 interact with a number of effector proteins. The best characterized effectors are the p21-activated kinases (PAKs). Both Rac and Cdc42 in the GTP-bound state interact specifically with PAKs and strongly stimulate PAK kinase activity (Manser *et al.*, 1994; Knaus *et al.*, 1995; Martin *et al.*, 1995). Mutants of Rac and Cdc42 that do not bind and activate PAK1 can still induce lamellipodia and filopodia, respectively (Joneson *et al.*, 1996; Lamarche *et al.*, 1996), however, activated PAK1 mutants themselves induce lamellipodia and cytoskeletal rearrangements (Sells *et al.*, 1997). Thus, the role of PAK1 in mediating effects of small GTPases on the cytoskeleton is presently unclear. In addition to effectors that are common to Rac and Cdc42, there are molecules such as WASP, POR-1, and p120ACK that interact with one or the other specifically, but the functions of these proteins are even less well defined (for review, see Tapon and Hall, 1997).

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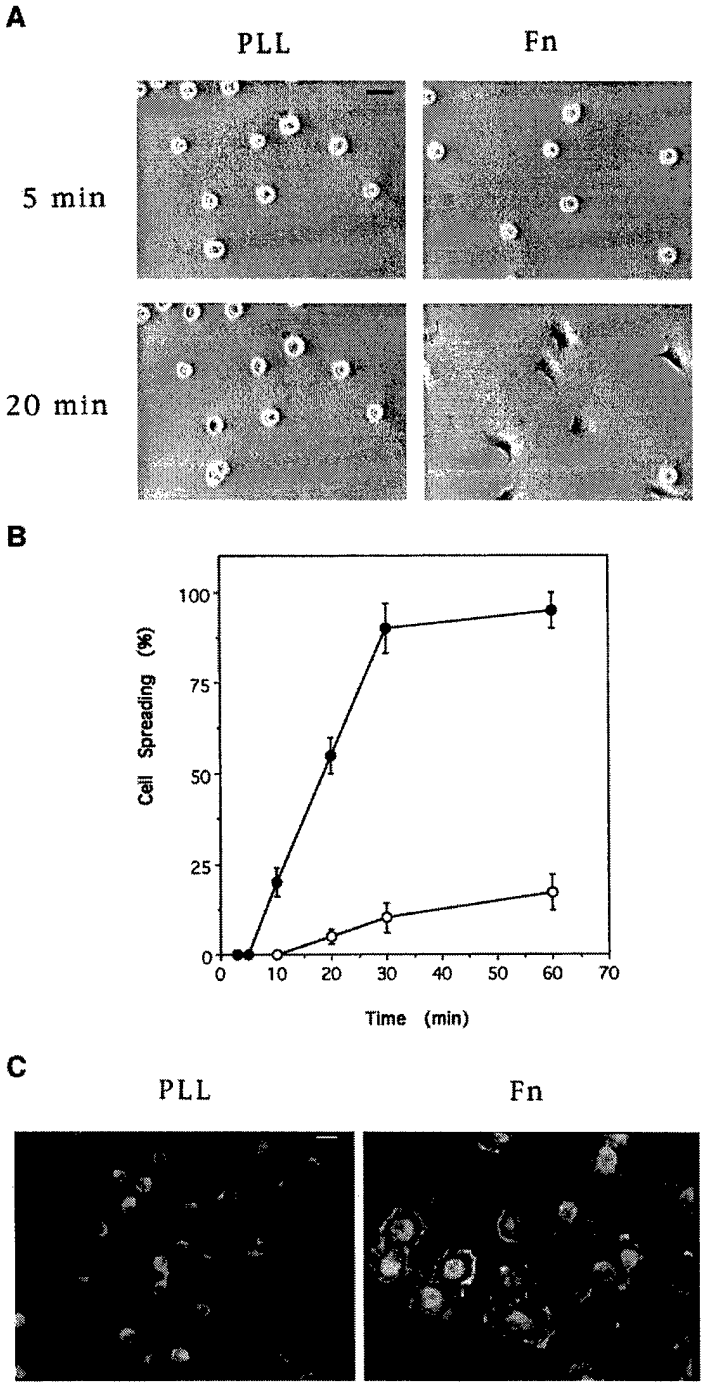

Figure 1. Cell spreading on Fn and poly-L-lysine. Quiescent cells in suspension were plated on coverslips coated with Fn or poly-L-lysine (PLL). Cells were fixed at the indicated times after plating and visualized by phase contrast microscopy (A) and scored for the percentage of spread cells (B) (●, Fn; ○, poly-L-lysine). (C) Twenty minutes after plating, cells were fixed and permeabilized, and filamentous actin was labeled with rhodamine–phalloidin; cells were then visualized by fluorescence microscopy. Bar, 20 μm.

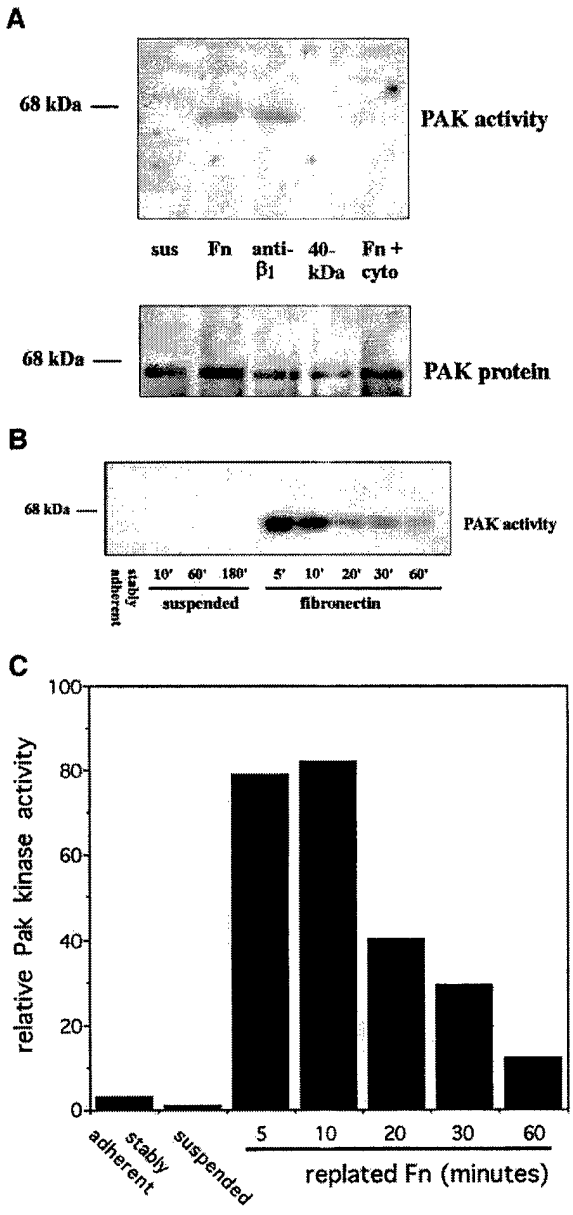

Figure 2. PAK kinase activity is stimulated after integrin-mediated cell adhesion. Cells were trypsinized, placed in suspension, and then plated on tissue culture dishes coated with the indicated ligands. PAK protein was immunoprecipitated, and its kinase activity was assayed. (A) Cells in suspension for 3 h (sus), replated for 20 min on Fn, anti-β1-integrin antibody, 40-kDa Fn fragment, or plated on Fn after a 30-min pretreatment with cytochalasin D (1 μM). The bottom panel shows the amount of PAK protein in immunoprecipitates as determined by Western blotting. (B) Stably adherent cells or cells suspended and replated on Fn for the indicated periods. (C) Densitometric quantification of B showing induction of kinase activity relative to suspended cells.

The similarity between cell spreading and Rac-induced lamellipodia formation prompted us to investigate the role of these small GTPases in cell spreading. Our results indicate that integrins activate these proteins and that both Rac and Cdc42 contribute to cell spreading.

MATERIALS AND METHODS

Proteins and Plasmids

Fibronectin (Fn) was purified from human plasma by affinity chromatography on gelatin-Sepharose (Miekka *et al.*, 1982). Fn 40-kDa α-chymotryptic fragment was purchased from Life Technologies (Gaithersburg, MD). The anti-β1-integrin antibody HMβ1-1 was purchased from PharMingen (San Diego, CA). Myelin basic protein (MBP) was purified from bovine spinal cord as described (Deibler *et al.*, 1972). NP-40 and leupeptin were purchased from ICN Biomedicals (Aurora, OH). Other chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Rac mutants were in a pcDNA3 vector; Cdc42 mutants were in pCMV5 (Zhang *et al.*, 1995); and PAK mutants were in pCMV6 (Sells *et al.*, 1997). The GFP vector was from Clontech (Palo Alto, Ca).

Microscopy

Cells were made quiescent by maintaining them in DMEM containing 0.5% serum for 24 h. Quiescent cells were trypsinized, washed, resuspended in serum-free DMEM, and plated on Fn- or poly-L-lysine-coated coverslips. Microscope images were collected continuously on a Panasonic video recorder. For quantification of spreading, cells were fixed in 3.7% formaldehyde at each time point, and the proportion of spread cells was determined under light microscopy. To visualize membrane ruffles, fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min, and actin filaments were stained with rhodamine–phalloidin (0.1 μg/ml) for 30 min. Coverslips were mounted in Fluoromount G and viewed by fluorescence microscopy.

Microinjection and Respreading

NIH 3T3 cells on coverslips were injected with cDNAs coding for various inhibitors of Cdc42 and Rac as described in the text. In some cases, DNA coding for GFP was included at 0.02 μg/ml to allow identification of injected cells. cDNAs were injected into the nucleus according to the method of Meredith *et al.* (1995). Dishes were returned to the incubator for 4 h to allow protein expression. Cells were incubated in trypsin-EDTA sufficiently to induce rounding without detachment, and then the trypsin was carefully aspirated, and fresh medium with 10% serum was added to stop the trypsin. Cells were returned to the incubator for 1 or 4 h as indicated and then fixed with 2% formaldehyde. They were stained for actin filaments with rhodamine–phalloidin (Molecular Probes, Eugene, OR) used at 0.1 μg/ml. Injected cells were identified either by GFP fluorescence or by staining for the myc-tagged dominant negative proteins with a monoclonal anti-myc antibody (9E10) and fluorescein-conjugated sheep anti-mouse secondary antibody. Both methods gave identical results.

Kinase Assays

To assay PAK kinase activity, 70% confluent NIH 3T3 fibroblasts were serum starved in DMEM with 0.5% bovine calf serum for 24 h. Where indicated, cells were trypsinized and suspended for three hours in serum-free DMEM containing 0.1% BSA (protease free) and 0.25 mg/ml soybean trypsin inhibitor. In some cases, cells were then pelleted by centrifugation, rinsed, and extracted in lysis buffer (20 mM Tris, pH 7.6, 0.5% NP-40, 250 mM NaCl, 5 mM EDTA, 3 mM

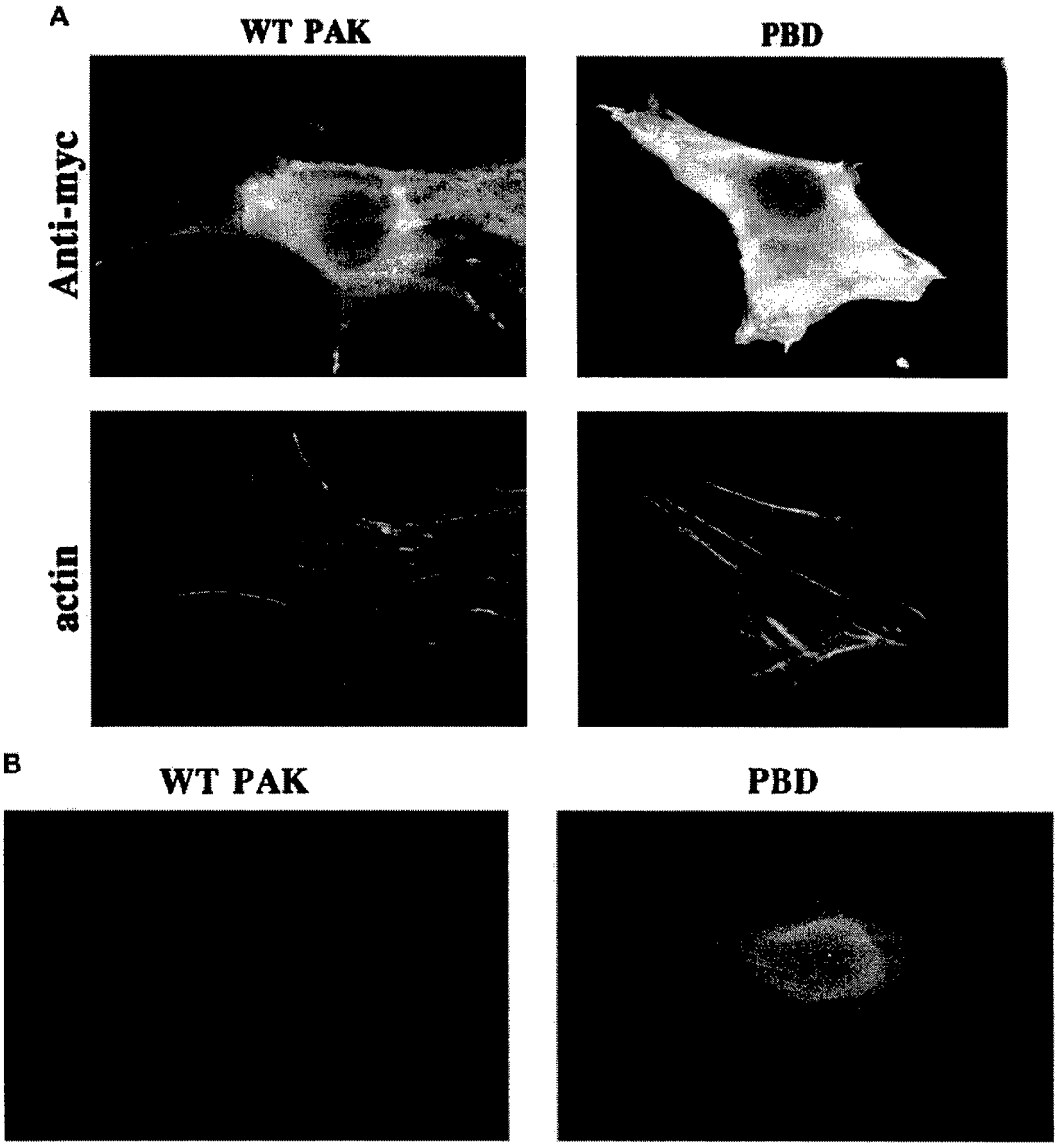

Figure 3. The PBD domain of PAK inhibits cell spreading. cDNAs of myc-tagged wild-type PAK (0.5 μg/ml) or the PBD domain of PAK (0.2 μg/ml) were injected into the nuclei of cells. (A) Labeling of expressed myc-tagged proteins with anti-myc antibody and of filamentous actin with rhodamine–phalloidin in stably spread cells after 4 h. (B) Respreading of injected cells for 1 h after mild trypsinization; expression of myc-tagged proteins is demonstrated by anti-myc staining. (C, facing page) Quantification of cell spreading. Values are means ± SD from three experiments in which >25 cells were scored per condition.

EGTA, 20 mM sodium phosphate, 10 mM sodium pyrophosphate, 3 mM β-glycerophosphate, 10 μg/ml leupeptin, 1 mM sodium vanadate, 1 mM PMSF, 1 mM NaF). Alternatively, cells were transferred to dishes that had been coated with 25 μg/ml Fn, anti-β1 immunoglobulin G, or the 40-kDa fragment of Fn and then blocked with 1% heat-denatured BSA. Cells were allowed to adhere for the indicated period, rinsed two times with cold PBS, and extracted in lysis buffer.

Endogenous PAK was immunoprecipitated from 150–250 μg cell lysate with anti-PAK1 antibodies (polyclonal anti-PAK1 R626; Dharmawardhane *et al.*, 1997) and dissolved in SDS-sample buffer. To estimate the amount of immunoprecipitated PAK, one-fifth of

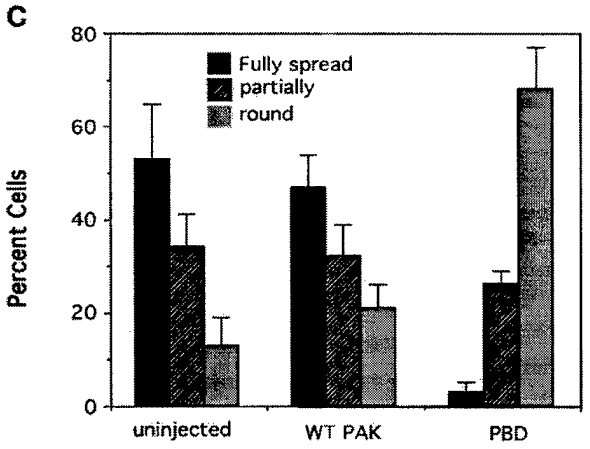

Figure 3 (cont).

each sample was run on 10% SDS-polyacrylamide gels and proteins transferred to nitrocellulose (Hybond C; Amersham, Little Chalfont, United Kingdom). Membranes were probed with the anti-PAK antibody, and protein was detected by enhanced chemiluminescence. PAK kinase activity in the immunoprecipitates was determined using an in-gel kinase assay as described previously (Renshaw *et al.*, 1996). Briefly, immunoprecipitates were run on 10% SDS-polyacrylamide gels containing 0.5 mg/ml MBP. Proteins in the gel were then renatured, and their kinase activity toward MBP was initiated by soaking the gels in kinase buffer containing 25 μCi/ml [γ-^{32}P]ATP and 10 μM unlabeled ATP. Gels were then washed extensively and autoradiographed, and films were scanned by densitometry using an Alphaimager 2000 (Alpha Innotech, San Leandro, CA).

RESULTS

Adhesion to Fn Stimulates Spreading and Membrane Ruffling

NIH 3T3 fibroblasts plated on Fn-coated surfaces adhere within ~5 min and then spread over a period of ~1 h (Figure 1, A and B). During this period, cells extend filopodia and lamellipodia and show extensive membrane ruffles (Figure 1C). Similar structures observed in growth factor-stimulated cells are mediated by the small GTPases Cdc42 and Rac, respectively (Ridley et al., 1992; Nobes and Hall, 1995). These results suggest that, in this cell type, Rac and or Cdc42 may be activated during cell spreading.

Integrin Activation of the Rac and Cdc42 Effector PAK

Direct assays of Rac or Cdc42 activation are technically difficult; therefore, to investigate possible integrin dependence of Rac and/or Cdc42 activity, we assayed the serine/threonine kinase PAK, which is a direct downstream target of these GTPases (Manser *et al.*, 1994; Knaus *et al.*, 1995). Cells were incubated for 24 h in 0.5% serum to minimize growth factor activation and then were detached and incubated in serum-free medium. After 3 h in suspension, cells had minimal PAK kinase activity, but plating on Fn-coated tissue culture dishes strongly stimulated PAK (Figure 2A). Activation was also observed when cells were plated on an antibody to β1-integrins but not on dishes coated with the 40-kDa tryptic fragment of Fn, to which cells adhere via heparin sulfate proteoglycans (Woods *et al.*, 1993). Consistent with the presence of PAK kinase activity, cells placed on Fn or anti-β1 antibodies extended processes and spread, whereas cells plated on the Fn 40-kDa fragment remained completely rounded (our unpublished results). Cell spreading and PAK activation were also observed when cells were plated on vitronectin. Pretreatment with cytochalasin D before plating cells on Fn prevented activation of PAK (Figure 2A), indicating that organization of the actin cytoskeleton is essential for integrin-mediated activation of this pathway. Cytochalasin D treatment also blocked cell spreading. These results demonstrate that integrin-dependent adhesion specifically activates PAK and, by inference, Rac and/or Cdc42.

Examination of the time course of PAK activation showed that kinase activity was nearly maximal at 5 min (the earliest time point measured), peaked at 10 min, and fell to a near-baseline level of activity after ~1 h (Figure 2, B and C). Thus, activation of this pathway is an early response to cell adhesion that precedes cell spreading.

Effect of Inhibitors of Rac and Cdc42 on Cell Spreading

To investigate whether activation of Rac and/or Cdc42 is required for cell spreading, cells were microinjected with cDNAs encoding epitope-tagged dominant negative mutant proteins, which inhibit these GTPases. We first examined the Rac and Cdc42 binding domain of PAK (p21 binding domain [PBD]) that binds and sequesters both Rac and Cdc42 and prevents their interaction with downstream effectors (Sells *et al.*, 1997). Expression of neither PBD nor wild-type PAK (which was used as a control) had any detectable effect on cell morphology or actin distribution in stably adherent cells after 4 h (Figure 3A). Expression of the proteins was confirmed by staining with an antibody against the epitope tag. To enable examination of spreading in microinjected cells, cells were induced to round up (but not detach) by brief trypsinization. The trypsin was stopped, and cells were allowed to respread for 1 h. Expression of wild-type PAK had no effect, but expression of PBD strongly inhibited respreading of cells in this assay (Figure 3, B and C). These results indicate that Rac and/or Cdc42 are required for cell spreading.

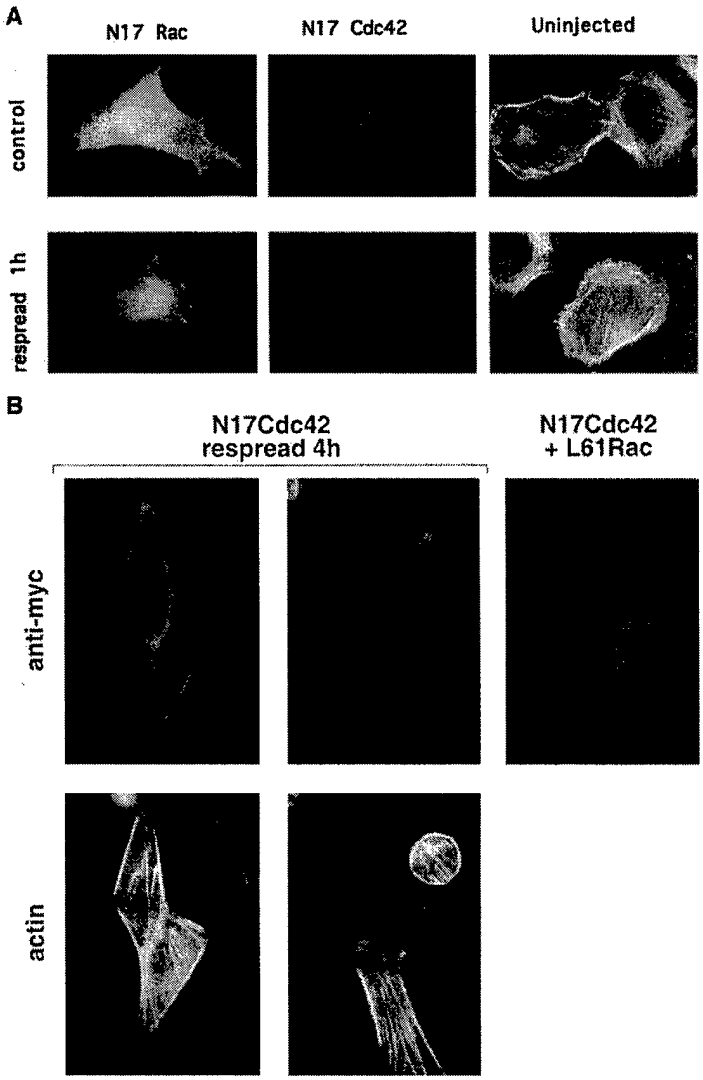

Figure 4. Effect of N17Rac and N17Cdc42 on cell spreading. N17Rac and N17Cdc42 cDNAs (0.2 μg/ml) were injected into the nuclei of cells. (A) Effect of expressed proteins on stably adherent cells (control) and cells allowed to respread for 1 h after mild trypsinization; anti-myc staining of injected cells and rhodamine–phalloidin staining of uninjected cells is shown. (B) Cells injected with N17Cdc42 and allowed to respread for 4 h or cells injected with N17Cdc42 plus constitutively active Rac (L61Rac) (0.5 μg/ml) and allowed to respread for 1 h. (C, facing page) Quantification of cell spreading. Values are means ± SD from four or five experiments in which >25 cells were scored per condition. The difference between N17 Rac and control cells is statistically significant ($p < 0.005$).

To determine to what extent Rac or Cdc42 or both mediate spreading, cells were injected with cDNAs encoding the dominant negative mutants N17Rac and N17Cdc42. Expression of these proteins in stably adherent cells did not cause gross changes in morphology or cytoskeletal structure, although N17Rac-expressing cells showed a loss of lamellipodia and an increase in filamentous projections, which were most likely either retraction fibers or filopodia (Figure 4A). Expression of N17Rac caused a partial inhibition of respreading of rounded cells (Figure 4, A and C). Notably, cells appeared to spread by means of narrow extensions instead of the usual broad lamellipodia. It should be noted that expression of fourfold lower levels of N17 Rac completely inhibited PDGF-induced ruffling, suggesting that the concentration of N17 Rac

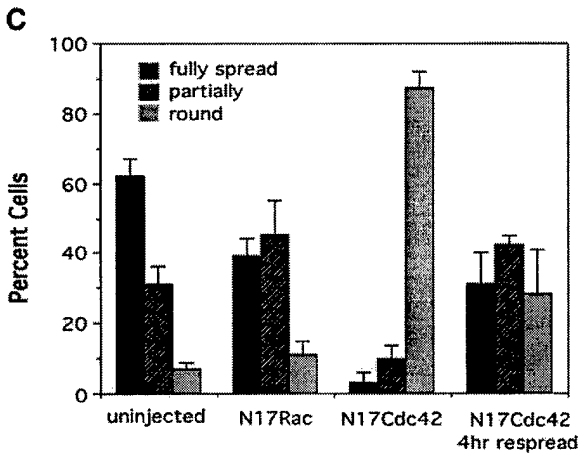

Figure 4 (cont).

DNA used in these experiments should be effective. By contrast, N17Cdc42 profoundly inhibited respreading; after 1 h, almost all injected cells remained completely round.

The inhibition by N17Cdc42 was so dramatic that additional controls were performed. To determine whether the inhibition of respreading by N17Cdc42 was reversible, cells were left to recover for longer periods. When cells were allowed to recover for 4 h, the majority of cells showed some degree of respreading (Figure 4, B and C). Interestingly, even those cells that failed to respread developed actin stress fibers (Figure 4B). Stress fibers have been shown to be a consequence of Rho activation, suggesting that Rho activation is independent of Cdc42. These results indicate that N17Cdc42-expressing cells remained viable and capable of assembling actin-based structures that are independent of Cdc42.

It has been demonstrated in some systems that Cdc42 can lead to activation of Rac (Nobes and Hall, 1995). Thus, the nearly complete inhibition of spreading by N17Cdc42 could be explained if initial activation of Cdc42 by integrins induced both Cdc42- and Rac-dependent events. To determine whether the inhibition of spreading by N17Cdc42-was due to the loss of Cdc42 activity alone or the additional loss of signaling to Rac, N17Cdc42 was expressed along with a constitutively activated mutant of Rac, L61Rac. Coexpression of activated Rac partially restored the ability of cells to respread after 1 h (Figure 4B). Quantification of these results showed that L61Rac increased the total number of spread cells (full plus partial) threefold from 15.2 ± 5.9 to 48.7 ± 18.1% ($p < 0.005$; $n = 5$). This result further demonstrates that N17Cdc42 is not toxic and suggests that Rac lies downstream of Cdc42 in this system.

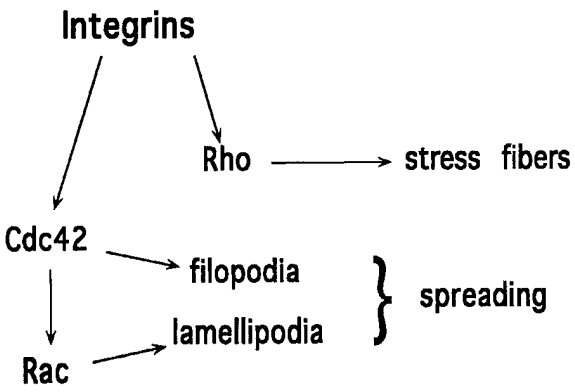

Figure 5. Schematic diagram showing integrin-dependent activation of Rho family GTPases. Engagement of integrins with ECM leads to the activation of Cdc42, which subsequently leads to the activation of Rac. Together, these GTPases mediate cell spreading. Activation of Rho can occur independently of activation of Cdc42 and leads to the formation of stress fibers.

DISCUSSION

Plating cells on Fn or antibodies to integrins leads to cell spreading and membrane ruffling. This result suggested that the small GTPase Rac was activated. We therefore investigated whether integrins activated Rac and whether this activation was involved in cell spreading.

The serine/threonine kinase PAK is activated directly by Rac and Cdc42 (Manser *et al.*, 1994; Knaus *et al.*, 1995; Martin *et al.*, 1995). We therefore assayed PAK kinase activity as an indicator of GTPase activation. PAK kinase activity was rapidly induced upon plating cells on Fn or anti-β1-integrin immunoglobulin G, suggesting that Rac and/or Cdc42 was indeed activated. PAK was not activated when cells adhered to a control protein that does not bind integrins, demonstrating a specific requirement for integrins. The time course of activation was rapid, with almost maximal kinase activity at 5 min. At this early time point, cell spreading is either minimal or absent, suggesting that Rac and/or Cdc42 activation precedes cell spreading.

To test whether Rac and Cdc42 were required for integrin-mediated cell spreading, we expressed the PBD domain of PAK, which inhibits these GTPases (Sells *et al.*, 1997). We found that the PBD domain profoundly inhibited cell spreading, demonstrating that Rac and/or Cdc42 GTPases are essential. To determine which of the two GTPases were required, we expressed dominant negative inhibitory forms of the proteins. We observed that dominant negative Rac retarded cell spreading, demonstrating a role for Rac. In the presence of N17Rac, cells spread by extending thin processes instead of lamellipodia, suggesting that

spreading occurred via Cdc42. Dominant negative Cdc42 profoundly inhibited spreading, with nearly all cells appearing completely round at 1 h, demonstrating a pivotal role for this protein. The deficiency in spreading in cells expressing N17Cdc42 could be overcome with time. This may have occurred via a Cdc42-independent mechanism, perhaps by the independent activation of Rac, or may be a consequence of incomplete inhibition of Cdc42. Interestingly, even in those cells that did not significantly spread, stress fibers still formed, indicating that Rho function was not dependent on Cdc42 or Rac.

To determine whether integrins activated Cdc42 and Rac independently or whether activation of these proteins was linear, we coexpressed N17Cdc42 with activated Rac. Activated Rac partially reversed the inhibition caused by N17Cdc42. As might be expected, the cells that spread did so by extending broad, symmetrical lamellipodia indicative of Rac activation (Ridley *et al.*, 1992). The incomplete recovery of spreading could be because Rac activation was not properly coordinated in space and time or because Cdc42 contributes something distinct from Rac that enhances spreading. Despite these uncertainties, the data suggest that integrin engagement leads to the activation of Cdc42, which results in the subsequent activation of Rac, and that both GTPases contribute to cell spreading.

Our results show that adhesion to ECM stimulates the activation of PAK. However, published data as to the role of PAK in Rac- and Cdc42-dependent control of cell architecture has been contradictory. PAK has been shown to colocalize with Rac and Cdc42 at focal adhesions and membrane ruffles (Harden *et al.*, 1996; Dharmawardhane *et al.*, 1997) and to induce filopodia and lamellipodia when injected into Swiss 3T3 cells (Sells *et al.*, 1997). Other studies, however, showed that mutations in Rac and Cdc42 that abolish binding to PAK in vitro did not block the ability to induce the formation of lamellipodia and filopodia (Joneson *et al.*, 1996; Lamarche *et al.*, 1996). To determine whether PAK is a functionally important effector in this system, the activated mutant PAK 83/86 (Sells et al., 1997) was coexpressed with N17 Cdc42. No recovery of cell spreading after trypsinization was observed, indicating that PAK is not sufficient for spreading in the absence of other effectors. These experiments do not, however, exclude the possibility that PAK could influence events triggered by other effectors. Thus, the function of PAK in GTPase-regulated cytoskeletal regulation is still unclear.

These results suggest a model for cell spreading whereby initial contact with ECM proteins activates Cdc42 and induces the extension of filopodial processes. Activation of Cdc42 leads to the subsequent activation of Rac and the formation of lamellipodia, which extend between the filopodia. Rho is activated independently to induce stress fibers and generate tension. This model is shown in Figure 5. It is notable that this model proposes a molecular mechanism for cell spreading that closely resembles cell migration, with the exception that instead of the unidirectional extension of filopodia and lamellipodia toward a chemotactic stimulus, processes extend isotropically to increase cell area.

We have shown here that integrins induce Rac- and Cdc42-mediated cell spreading in the absence of exogenous growth factors. By contrast, adherent serum-starved Swiss 3T3 cells do not possess stress fibers or membrane ruffles and require growth factors or addition of activated Rho and Rac to induce these structures (Ridley and Hall, 1992; Ridley *et al.*, 1992). These differences may be due to basic differences in the signaling pathways between the two cell types. However, it has been shown that if the level of integrin occupancy in starved Swiss 3T3 cells is increased by replating cells on Fn or by adding the Fn cell-binding peptide GRGDS, cells do spread and form Rho-dependent stress fibers (Allen *et al.*, 1997). These results suggest that it is primarily the extent of integrin occupancy required to induce activation of GTPases that differs between the two cell types.

Adhesion is a basic requirement for proliferation of most cell types. It has been proposed that constitutive activation of signaling pathways that are normally activated by integrins may overcome the adhesion requirement for cell growth (Schwartz, 1993; Schwartz *et al.*, 1996). Recent results showing that activated Cdc42 confers anchorage-independent growth but that cells remain serum dependent (Qiu *et al.*, 1997) therefore support our conclusion that integrins activate Cdc42. These results further suggest that integrin-mediated adhesion may therefore contribute to cell proliferation via the activation of Cdc42.

Evidence has also been presented that Rho is activated by integrins and that enhanced activation of Rho by overexpression of Rho nucleotide exchange factors can also confer anchorage independence (Chong *et al.*, 1994; Schwartz *et al.*, 1996). How integrins activate Cdc42 and Rho is not known, but the most obvious hypothesis is that they may activate nucleotide exchange factors for these GTPases. Although not yet identified in NIH 3T3 cells, exchange factors have been identified in other cell types that act on Cdc42 and Rho (Horii *et al.*, 1994; Olson *et al.*, 1996). Regulation of these factors by integrins may therefore be a useful direction for future investigations.

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CAS/Crk Coupling Serves as a "Molecular Switch" for Induction of Cell Migration

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Abstract. Carcinoma cells selected for their ability to migrate in vitro showed enhanced invasive properties in vivo. Associated with this induction of migration was the anchorage-dependent phosphorylation of p130CAS (Crk-associated substrate), leading to its coupling to the adaptor protein c-CrkII (Crk). In fact, expression of CAS or its adaptor protein partner Crk was sufficient to promote cell migration, and this depended on CAS tyrosine phosphorylation facilitating an SH2-mediated complex with Crk. Cytokine-stimulated cell migration was blocked by CAS lacking the Crk binding site or Crk containing a mutant SH2 domain. This migration response was characterized by CAS/Crk localization to membrane ruffles and blocked by the dominant-negative GTPase, Rac, but not Ras. Thus, CAS/Crk assembly serves as a "molecular switch" for the induction of cell migration and appears to contribute to the invasive property of tumors.

During wound repair and inflammation, cells can be induced to migrate by cytokines or adhesive proteins within the provisional extracellular matrix (ECM)[1] (Clark et al., 1996). Recent evidence indicates that cytokine receptors as well as integrins transmit signals in response to extracellular cues (for reviews see Schwartz et al., 1995; Parsons, 1996). While these biochemical signals are likely to be involved in the induction of cell migration, little is known about the actual signal transduction events responsible for this process or how they impact the cells' migration machinery.

Ultimately, the biochemical signals responsible for controlling cell migration must impact adhesion and deadhesion of integrin receptors as well as organization of the actin and myosin cytoskeleton since these events are critical for movement (Huttenlocher et al., 1996). However, the complexity of cell motility suggests that multiple signaling mechanisms exist to regulate this process. For example, Ras/MAP kinase (ERK1 and 2) signaling has been shown to promote phosphorylation of myosin light chain kinase leading to activation of the actin/myosin motor and cell migration (Klemke et al., 1997), whereas the Rho family of small GTPases (i.e., Rac, Rho, Cdc42) control actin organization associated with cell motility (Ridley et al., 1992; Takaishi et al., 1993; Hotchin and Hall, 1995; Michiels et al., 1995). Furthermore, the adhesion-dependent signaling molecules focal adhesion kinase (FAK) and c-src may be involved in focal contact disassembly during cell migration (Ilic et al., 1995; Rodier et al., 1995; Cary et al., 1996; Gilmore and Romer, 1996; Parsons, 1996; Hanks and Polte, 1997). Phosphatidylinositol 3-kinase (Rodriguez-Viciana et al., 1997) and PLCγ (Chen et al., 1994) are also thought to regulate cell migration. Therefore, it is likely that cell migration depends on the coordinate regulation of a number of these signaling events.

Ligation of integrin or cytokine receptors promote a cascade of biochemical signals, including the activation of tyrosine kinases leading to phosphorylation of multiple cellular substrates. A family of adaptor proteins (Nck, Crk, Grb2), which consist primarily of src homology 2 (SH2) and 3 (SH3) domains, coordinate these biochemical events by assembling signal-generating complexes. For example, the SH2 domains of these adaptor proteins bind specifically to phosphotyrosine-binding sites in many signaling molecules, while the SH3 domains are critical for coupling to effector molecules and targeting of signaling complexes to discrete sites within the cell (Bar-Sagi et al., 1993).

The adaptor protein p130CAS (Crk-associated substrate) is a member of a family of structurally related proteins (i.e., Efs/Sin and Hef 1) that was originally identified

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1. *Abbreviations used in this paper*: aa, amino acid(s); CAS, Crk-associated substrate; ECM, extracellular matrix; FAK, focal adhesion kinase; FBM, fibroblast basal medium; IGF-1, insulin-like growth factor 1; SH, src homology.

as a prominent tyrosine-phosphorylated protein in v-crk– and v-src–transformed cells (Sakai et al., 1994; Greulich and Hanafusa, 1996; Matsuda and Kurata, 1996). CAS contains an SH3 domain, two proline-rich regions, and a substrate domain consisting of 15 potential SH2-binding motifs (Sakai et al., 1994). C-Crk II (Crk) is an adaptor protein that contains an SH2 and two SH3 domains capable of interacting with numerous effector molecules, including tyrosine-phosphorylated CAS (for reviews see Feller et al., 1994*a*; Matsuda and Kurata, 1996). In fact, 9 of the 15 tyrosine phosphorylation sites present in the substrate domain of CAS conform to the SH2-binding motif for Crk (YD(V/T)P, suggesting that Crk is the primary "docking protein" for CAS. However, Nck also binds to CAS and may play a role in regulation of CAS activity (Schlaepfer et al., 1997). Recently, it was reported that cell adhesion to ECM proteins promotes FAK and c-src kinase activity leading to tyrosine phosphorylation of CAS and its association with Crk or Nck (Vuori et al., 1996; Schlaepfer et al., 1997). While this suggests a role for these molecules in mediating cellular responses to the ECM, the biological consequences of this signaling event are not known.

In this report, we investigate the role of CAS and Crk in regulation of cell migration on the ECM. Evidence is provided that tyrosine phosphorylation of CAS is associated with induction of migration and enhanced invasive potential of carcinoma cells in vivo. This migration response was shown to depend on the assembly of a CAS/Crk adaptor protein complex, and the resulting migration was disrupted by a dominant-negative form of Rac, but not Ras. These findings suggest that formation of a CAS/Crk adaptor protein complex serves as a "molecular switch" facilitating a Rac-dependent cell migration response on the extracellular matrix.

Materials and Methods

Cells and Cell Culture

Previous reports have described the FG human pancreatic carcinoma cell adhesive and migratory properties and integrin profile (Klemke et al., 1994). FG-M is a cell line derived from FG cells that are migration competent on vitronectin. To obtain these cells, FG cells were serum starved for 48 h before being removed from the culture dish with Hanks' balanced salt solution containing 5 mM EDTA and 25 mM Hepes, pH 7.2, and allowed to migrate for 24 h on vitronectin-coated (10 μg/ml in PBS, pH 7.4) migration wells as previously reported (Klemke et al., 1994). The migratory cells on the underside of the membrane were removed with trypsin/EDTA solution (GIBCO BRL, Gaithersburg, MD) and cultured in RPMI 10% FCS until enough cells were obtained to repeat the process. After 10 rounds of selection, we obtained a stable cell line (FG-M cells) that spontaneously migrates on vitronectin to levels comparable to that of parental FG cells on collagen. FG and FG-M cells were grown in RPMI 1640 (GIBCO BRL) supplemented with 10% FBS and 50 μg/ml gentamicin and were free from mycoplasma during these studies. COS-7 cells were grown in DME with 10% FBS and 50 μg/ml gentamicin. Before testing, all cells were starved for 24–48 h by replacing serum-containing culture media with FBS-free media.

Expression Vectors and Constructs

The expression plasmid pUCCAGGS containing full-length CrkII as well as c-CrkII cDNA in which either the amino-terminal SH3 domain (tryptophan 109 to cysteine) or the SH2 domain (arginine 38 to valine) were mutated have been described previously (Matsuda et al., 1993, 1994; Tanaka et al., 1993). The pEBG expression plasmid containing glutathione-S-transferase (gst)-tagged or untagged wild-type p130CAS or CAS with an in frame deletion of its substrate domain (CAS-SD, amino acids [aa] 213–514) has been described previously (Mayer et al., 1995). Myc-tagged dominant-positive Racl (Q61L) or dominant-negative Rac 1 (N17) in pcDNA3, and dominant-negative Ras (N17) in pCMV5 vectors have been described previously (Minden et al., 1995).

Antibodies and Reagents

Anti-Crk was from Transduction Laboratories (Lexington, KY). Anti-CAS used for Western blotting and immunofluorescent staining was from Transduction Laboratories, and anti-CAS antibodies for immunoprecipitation experiments were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-myc antibody 9E10 and anti-Ras (259) was from Santa Cruz Biotechnology. Anti-gst and antiphosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology, Inc. (Lake Placid, NY). Goat anti–rabbit and anti–mouse antibodies were from Bio-Rad Labs (Hercules, CA). Anti-FAK antibody BC3 was kindly provided by Dr. J. Thomas Parsons (University of Virginia Medical Center, Charlottesville, VA). Human recombinant EGF, IGF-1, and insulin were obtained from Genzyme (Cambridge, MA).

Adhesive Ligands

Vitronectin was prepared as described by Yatohgo et al. (1988). Fibronectin and collagen type I were obtained from Upstate Biotechnology, Inc.

Transfection of COS-7 and FG -M Cells

Transient transfection of COS cells was performed as previously described (Klemke et al., 1997). Briefly, to investigate the role of CAS or Crk in cell migration, COS-7 cells (0.75×10^6 cells/10-cm plate) were cotransfected with lipofectamine (20 μl/10-cm plate; GIBCO BRL) and 1.5 μg of the expression vector containing the cDNA encoding wild-type and/or mutant forms of c-Crk or CAS, along with 0.5 μg of a reporter construct encoding β-galactosidase (pSV–β-galactosidase; Promega Corp., Madison, WI). In some cases, cells were transfected with the expression vectors encoding wild-type CAS and Crk (1.5 μg of each construct per plate) along with dominant-negative Rac construct (1 μg/plate) or Ras (0.25 μg/plate) as described above. Mock cells were transfected as described above with the appropriate amount of the empty expression vectors. Cells were allowed to incorporate the cDNA constructs for 6–8 h, washed, and then allowed to incubate for 40 h, which provides optimal transient expression. FG-M cells (1×10^6 cells/10-cm dish) were cotransfected with lipofectamine (40 μl/10-cm plate) and 10 μg of the expression vector containing CAS without its substrate domain (CAS-SD) or the empty expression vector along with the β-galalctosidase reporter construct (10 μg/10-cm plate). Cells were allowed to incorporate the cDNAs for 16 h, washed, and then incubated for 36 h. COS and FG-M cells were then prepared for cell migration or analyzed for expression of specific proteins as described below. Cells cotransfected with β-galactosidase were developed using X-gal as a substrate according to the manufacturer's recommendation (Promega Corp.). In typical transfection experiments of FG-M cells, we achieved 10–15% expression efficiency, and in COS-7 cells we obtained 40–60% efficiency providing a 10–30-fold increase in the level of Crk, 10–15-fold increase in CAS, or 10–30-fold increase in Rac and Ras compared with endogenous levels of these proteins as determined by Western blotting.

Haptotaxis Migration Assays

Cell adhesion and migration assays were performed as previously described with minor modifications (Klemke et al., 1997). Briefly, cell migration assays were performed using modified Boyden chambers (tissue culture–treated, 6.5-mm diameter, 10-μm thickness, 8-μm pores, Transwell®; Costar Corp., Cambridge, MA) containing polycarbonate membranes coated on the underside of the membrane with 10 μg/ml vitronectin, fibronectin, or collagen type I in PBS for 2 h at 37°C, rinsed once with PBS, and then placed into the lower chamber containing 500 μl migration buffer (fibroblast basal medium [FBM] with 0.5% BSA; Clonetics, San Diego, CA). Serum-starved cells were removed from culture dishes with Hanks' balanced salt solution containing 5 mM EDTA and 25 mM Hepes, pH 7.2, and 0.01% trypsin, washed twice with migration buffer, and then resuspended in FBM 0.5% BSA (10^6 cells/ml). 50,000–100,000 cells were then added to the top of each migration chamber and allowed to migrate to the underside of the top chamber for various times in the presence or

absence of either insulin (25 μg/ml), insulin-like growth factor 1 (IGF-1; 20 ng/ml), or epidermal growth factor (50 ng/ml), which had been added to the lower chamber. The nonmigratory cells on the upper membrane surface were removed with a cotton swab, and the migratory cells attached to the bottom surface of the membrane stained with X-gal substrate as described above (for β-galactosidase–expressing cells) or 0.1% crystal violet in 0.1 M borate, pH 9.0, and 2% ethanol for 20 min at room temperature. The number of migratory cells per membrane were either counted with an inverted microscope using a 40× objective, or the stain was eluted with 10% acetic acid, the absorbance was determined at 600 nm, and migration was enumerated from a standard curve. Each determination represents the average of three individual wells, and error bars represent the standard deviation (SD). All values have had background subtracted, which represents cell migration on membranes coated on the bottom with BSA (1%). In control experiments, cell migration on BSA was less than 0.01% of the total cell population.

Chick Embryo Metastasis Assay

The chick embryo metastasis assay was performed as previously described (Brooks et al., 1997). Briefly, FG or FG-M carcinoma cells ($5–10 \times 10^6$) were inoculated on the back of the chorioallantoic membrane of a 9-d-old chick embryo and allowed to incubate for 7–9 d. Embryos were then killed, tumors were excised, and the weights were determined. Pulmonary metastasis was quantified by determining the percentage of human cells present in a single cell suspension of whole lung tissue by flow cytometry as described by Brooks et al. (1997).

Cell Adhesion and Transfection Efficiency

Cell adhesion was performed according to Klemke et al. (1994) with minor modifications. Briefly, 48-well cluster plates (polystyrene, non-tissue culture–treated; Costar, Cambridge, MA) were coated with either 10 μg/ml vitronectin, fibronectin, or collagen 1 in PBS, pH 7.4. Proteins were allowed to bind for 2 h at 37°C before the wells were rinsed and blocked for 1 h with 1% heat-denatured BSA (RIA grade; Sigma Chemical Co., St. Louis, MO) in PBS. Cells were treated and harvested as for the migration assay and added to the wells at a concentration of 100,000 cells/0.1 ml FBM-BSA. Nonadherent cells were removed by washing, and the adherent cells were stained with crystal violet and enumerated from dye uptake or stained with X-gal substrate and counted as described above. Each data point was calculated from triplicate wells and was expressed as the mean ± SD. Nonspecific cell adhesion as measured on BSA-coated wells has been subtracted.

Immunoprecipitation and Immunoblotting of Proteins

Immunoprecipitation and immunoblotting of proteins was performed as previously described (Klemke et al., 1997). Briefly, cells were rinsed twice with PBS and then lysed with either RIPA buffer for Western blotting of total cellular proteins or NP-40 buffer (50 mM Tris, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, a cocktail of protease inhibitors [Boehringer Mannheim cocktail tablet; Boehringer Mannheim Corp., Indianapolis, IN], and 1% NP-40) for immunoprecipitation of cellular proteins for 1 h at 4°C. The protein concentration of the samples were normalized before either direct immunoblotting of total cell proteins or immunoprecipitation of equivalent amounts of proteins with Crk, CAS, or gst antibodies bound to protein A– or G–Sepharose beads. Samples were analyzed by immunoblot analysis using antibodies to Crk, CAS, gst, or antiphosphotyrosine monoclonal antibody (4G10) and horseradish peroxidase–conjugated goat anti–mouse or rabbit secondary antibodies and the ECL system. In some cases, the immunoblots were stripped and reprobed with antibodies according to the manufacturer's recommendations (ECL; Amersham Corp., Arlington Heights, IL).

Immunofluorescent Staining and Confocal Microscopy

COS-7 cells either transfected with CAS and Crk or mock-transfected cells were removed from the culture dish and resuspended in FBM (0.5% BSA) as described above and allowed to attach to glass coverslips coated with 10 μg/ml vitronectin for 2 h at 37 °C. In some cases, cells grown on glass coverslips were serum-starved for 24 h and then treated with or without insulin (25 μg/ml) or IGF-1 (20 ng/ml) for 15 min. Cells were fixed in 4% paraformaldehyde, permeablized with 0.2% Triton X-100, and stained with rhodamine-conjugated phalloidin and anti-Crk or anti-CAS antibodies followed by FITC-conjugated goat anti–mouse or rabbit secondary antibodies. Cell fluorescence was analyzed with a laser confocal microscope (model 1024; Bio-Rad Labs) and a Zeiss Axiovert microscope (Thornwood, NY) focused at the cell–substratum interface.

Results

Selection of FG Pancreatic Carcinoma Cells with Increased Migration In Vitro and Metastatic Properties In Vivo

While cell adhesion to the extracellular matrix is required for cell migration, it is not sufficient. For example, FG pancreatic carcinoma cells attach to vitronectin but fail to migrate on this adhesive ligand, whereas, they readily migrate on collagen (Klemke et al., 1994). This may be explained by differences in adhesion-dependent signaling events that ultimately facilitate cell movement. To identify putative signaling molecules that are associated with the migratory phenotype, FG cells were allowed to attach to either collagen or vitronectin and examined for differences in their phosphoprotein profile. As shown in Fig. 1, cell attachment to collagen, a migration-competent ligand, resulted in significantly enhanced tyrosine phosphorylation of a 70- and 125–130-kD protein(s), whereas, cell adhesion to vitronectin, which does not support FG cell migration, showed only little tyrosine phosphorylation of these proteins.

To determine whether the phosphorylation status of the 125–130-kD phosphoprotein(s) was associated with the migratory phenotype of these cells, we first selected a stable population of FG cells that were migration competent on vitronectin since a small percentage of the parental FG cells (<0.05%) were capable of movement on this substrate. After 10 rounds of selection, a stable population was

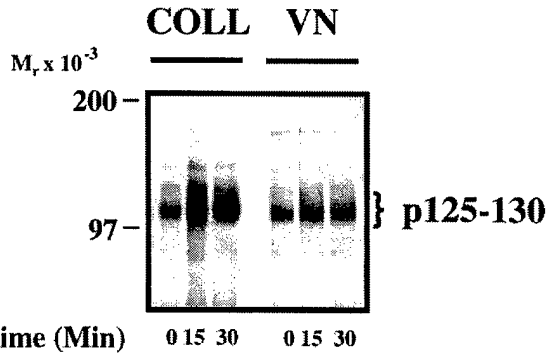

Figure 1. FG cell migration on collagen is associated with increased tyrosine phosphorylation of p125–130 proteins. Serum-starved FG cells were held in suspension (0 time point) or allowed to attach to Petri dishes coated with collagen (*COLL*) or vitronectin (*VN*) for various times before being lysed and examined for changes in tyrosine phosphorylation of total cellular proteins by phosphotyrosine immunoblotting as described in Materials and Methods. Cell migration was determined in modified Boyden chambers (Transwells) coated with either collagen or vitronectin as described in Materials and Methods. Cell migration on collagen (+) was greater than 50% of the cell population in 24 h, while on vitronectin less than 1.0% of the cells migrated.

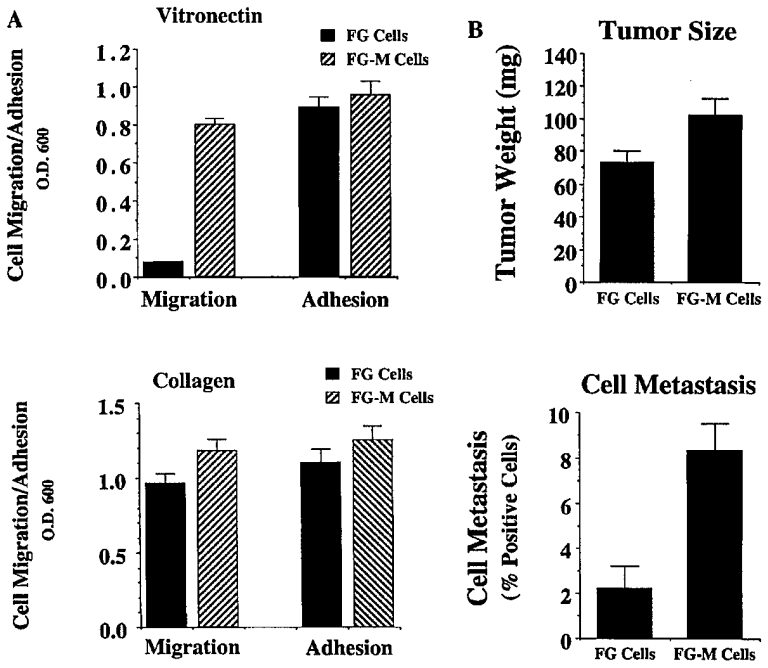

Figure 2. Comparison of FG and FG-M cell migration in vitro and metastasis in vivo. (*A*) FG-M cells are a stable cell line derived from FG cells selected for their ability to migrate on a vitronectin substrate as described in Materials and Methods. Cells were allowed to adhere for 30 min to Petri dishes or migrate for 4 h on Transwell migration chambers coated with vitronectin (*top*) or collagen (*bottom*) and quantified as described in Materials and Methods. Each bar represents the mean ± SD of triplicate migration wells of one of three representative experiments. (*B*) FG or FG-M carcinoma cells (5×10^6) inoculated onto chorioallantoic membrane of 9-d-old chick embryos were allowed to form tumors for 9–10 d, at which time the tumors were resected and weighed (*top*). Pulmonary metastasis (*bottom*) was measured as described in Materials and Methods. Tumor weight is the mean ± SEM. Percent positive metastasis is the mean ± SEM of the relative percentage of tumor cells in the lungs of the chick embryos. FG-M cells showed a statistically significant increase in metastasis over FG cells ($P < 0.01$) as determined by Student's *t* test.

obtained (FG-M cells) that showed spontaneous motility on vitronectin that was comparable to FG cell migration on collagen (Fig. 2 *A*). While FG-M cells gained the ability to migrate on vitronectin, there was no difference in their integrin profile or their capacity to adhere to vitronectin (Fig. 2 *A*) or a number of other extracellular matrix proteins (data not shown). Therefore, FG-M cells represent a stable migratory subpopulation of the parental FG cells.

Cell migration on vitronectin in vitro has been linked to the metastatic capacity of tumor cells in vivo (Nip et al., 1992; Brooks et al., 1997). Therefore, we compared FG and FG-M cells for their ability to metastasize spontaneously from the chick chorioallantoic membrane to the lungs of 10-d-old chick embryos. As shown in Fig. 2 *B* (*bottom*), FG-M cells showed a fourfold increase in spontaneous metastasis compared with FG cells. However, there was only a slight increase in the average FG-M primary tumor weight (1.4-fold) compared with primary tumors formed by FG cells (Fig. 2 *B*, *top*). Furthermore, FG and FG-M cells had identical growth rates in vitro (data not shown). These findings suggest that the enhanced migratory properties of FG-M cells in vitro are primarily associated with their increased metastatic properties in vivo.

FG-M Cells Show Increased CAS Tyrosine Phosphorylation

To establish a possible role of the p125–130 protein(s) in the migratory capacity of FG-M cells, we compared the phosphoprotein profile of FG and FG-M cells during their attachment to vitronectin. As shown in Fig. 3 *A*, FG-M, but not FG cell attachment to vitronectin, resulted in the tyrosine phosphorylation of proteins with a molecular mass of 125–130 kD. This phosphorylation pattern appeared similar to what was observed between FG cells attached to collagen vs. vitronectin and thus appeared to be associated with FG cell migration in general (Fig. 1). Together, these data demonstrate that cells selected for spontaneous migration in vitro show enhanced invasive properties in vivo as well as adhesion-dependent phosphorylation of proteins with a molecular mass of 125–130 kD.

Previous studies have demonstrated that p125 FAK and p130CAS are phosphorylated upon cell adhesion to matrix proteins (Ilic et al., 1995; Nojima et al., 1995; Vuori and Ruoslahti, 1995; Hanks and Polte, 1997). Recently, FAK has been linked to cell migration since cells lacking this protein show reduced cell migration in vitro (Ilic et al., 1995). Therefore, we investigated the possibility that FAK was a component of the p125–130-kD proteins phosphorylated in the FG-M cells. FAK was immunoprecipitated from vitronectin-attached FG or FG-M cells. The immunoprecipitated proteins were then analyzed for the presence of phosphotyrosine by immunoblotting. Interestingly, both FG and FG-M cells showed a similar increase in FAK tyrosine phosphorylation upon adhesion to vitronectin (Fig. 3 *B*). Importantly, FAK phosphorylation was not readily detectable in total cell lysates prepared from these cells since they express low levels of this protein (data not shown). While tyrosine phosphorylation of FAK appeared identical in these cells, CAS, immunoprecipitated from FG-M cells attached to vitronectin, was highly tyrosine-phosphorylated compared with CAS precipitated from FG cells (Fig. 3 *C*). However, CAS immunoprecipitated from FG cells attached to collagen was highly tyrosine phosphorylated, indicating that these cells have the capacity to phosphorylate this protein (data not shown). In either case, the enhanced phosphorylation was not the result of an increase in CAS expression since simi-

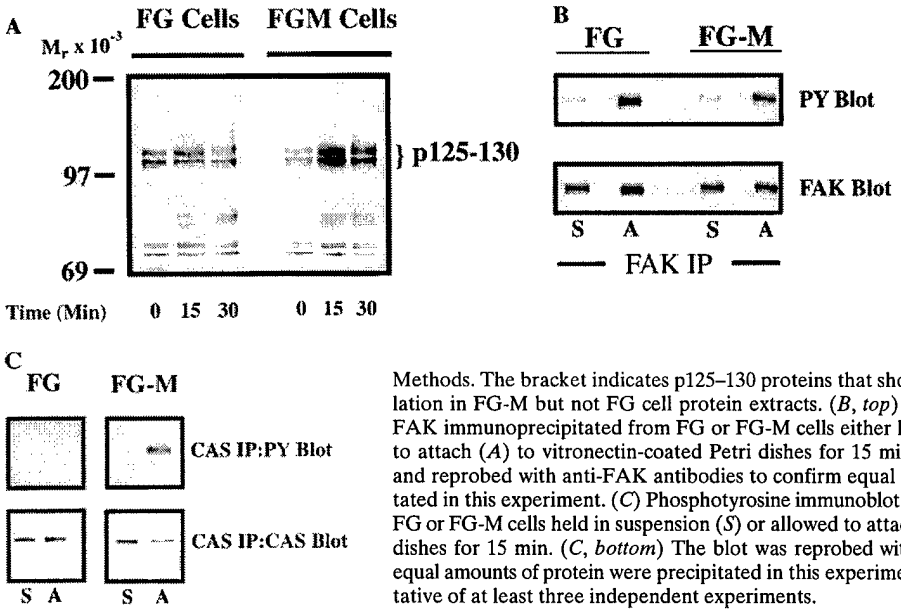

Figure 3. FG-M, but not FG cells, show increased tyrosine phosphorylation of p130CAS upon attachment to vitronectin. (*A*) Serum-starved FG or FG-M cells were held in suspension or allowed to attach to vitronectin-coated Petri dishes for various times before being lysed in detergent and analyzed for changes in tyrosine phosphorylation of total cellular proteins as described in Materials and Methods. The bracket indicates p125–130 proteins that show increased tyrosine phosphorylation in FG-M but not FG cell protein extracts. (*B*, *top*) Phosphotyrosine immunoblot of FAK immunoprecipitated from FG or FG-M cells either held in suspension (*S*) or allowed to attach (*A*) to vitronectin-coated Petri dishes for 15 min. (*B*, *bottom*) Blot was stripped and reprobed with anti-FAK antibodies to confirm equal amounts of protein were precipitated in this experiment. (*C*) Phosphotyrosine immunoblot of CAS immunoprecipitated from FG or FG-M cells held in suspension (*S*) or allowed to attach (*A*) to vitronectin-coated Petri dishes for 15 min. (*C*, *bottom*) The blot was reprobed with anti-CAS antibodies to confirm equal amounts of protein were precipitated in this experiment. The result shown is representative of at least three independent experiments.

lar amounts of this protein were immunoprecipitated from both cell types (Fig. 3 *C*). These findings suggest that phosphorylation of CAS may be associated with the migratory properties of these cells.

Expression of CAS in Cells Is Sufficient to Promote Cell Migration on the ECM

To establish whether CAS played a direct role in cell motility, we expressed the wild-type form of this molecule in COS cells since these cells efficiently express proteins encoded by eukaryotic expression vectors and show negligible migration on several ECM proteins after serum starvation (Klemke et al., 1997). Transient expression of CAS in these serum-starved cells promoted a fourfold increase in their migration on vitronectin (Fig. 4 *A*, *top*) as well as on collagen and fibronectin (data not shown). Associated with this induction of migration was increased tyrosine phosphorylation of both the expressed CAS (Fig. 4 *A*, *bottom*) and endogenous CAS protein (data not shown). Importantly, expression of CAS did not alter the ability of these cells to attach to any of the substrates tested (data not shown), suggesting that CAS expression and its associated phosphorylation is sufficient to promote cell migration.

The substrate domain of CAS contains most of the putative tyrosine phosphorylation sites in this molecule (tyr-377 to tyr-414) and accounts for a significant portion of its tyrosine phosphorylation (Sakai et al., 1994). To examine the role of the CAS substrate domain in CAS-mediated migration, cells were transfected with either wild-type CAS or CAS containing an in-frame deletion of its substrate domain (aa 213–514; CAS-SD). As shown in Fig. 4 *A* (*top*), cells expressing CAS-SD failed to migrate and did not show CAS tyrosine phosphorylation in response to cell attachment (Fig. 4 *A*, *middle*). In addition, transfection of FG-M cells with CAS-SD reduced their migration on vitronectin and collagen substrates by >50%. (Fig. 4 *B*). These findings provide further evidence that CAS signaling is involved in cell migration and suggests that CAS tyrosine phosphorylation of its substrate domain is required for CAS-induced migration.

Crk Binding to CAS Is Required for the Induction of Cell Migration

Tyrosine phosphorylation of CAS within its substrate domain is known to promote coupling to the adaptor protein Crk (Matsuda et al., 1993; Feller et al., 1994*b*). In fact, 9 of the 15 tyrosine phosphorylation sites in the CAS substrate domain conform to the Crk SH2–binding motif (YD (V/T)P. To investigate the possibility that CAS binding to Crk was involved in promoting cell migration, COS cells were transfected with Crk and either wild-type CAS or CAS-SD and then allowed to migrate on a vitronectin substrate after serum starvation. As shown in Fig. 5 *A* (*top*), expression of either wild-type CAS or Crk promoted a three- to fourfold increase in cell migration compared with cells expressing an empty vector. Interestingly, coexpression of both CAS and Crk in these cells further enhanced their migratory properties resulting in a seven- to eightfold increase in cell migration (Fig. 5 *A*, *top*). However, when Crk was expressed together with CAS-SD, motility was blocked (Fig. 5 *A*). Similar results were obtained when cells were allowed to migrate on collagen and fibronectin, and expression of these cDNAs did not influence the ability of these cells to attach to any of these matrix proteins (data not shown). In addition, stable expression of Crk in FG cells was sufficient to promote a 10-fold increase in cell migration on vitronectin compared with mock-transfected cells (data not shown). Together, these results suggest that the interaction between Crk and CAS is critical for migration on multiple ECM proteins but not attachment of these

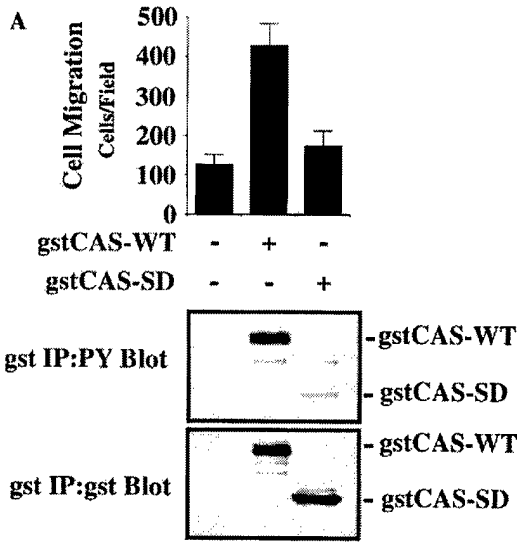

Figure 4. Expression of CAS in cells is sufficient to induce cell migration. (*A*, *top*) Serum-starved COS cells were allowed to migrate for 3 h on vitronectin-coated membranes after transient transfection with either the empty expression vector or the expression vector containing gst-tagged wild-type CAS (*gstCAS-WT*) or gst-tagged CAS lacking a substrate domain (i.e., aa 213–514 and tyr-377 to tyr-414; *gstCAS-SD*). The number of transfected cells migrating were enumerated by counting cells on the underside of the membrane that coexpressed a β-galactosidase vector as described in Materials and Methods. Each bar represents the mean ± SD of triplicate migration wells of one of three representative experiments. Identical results were obtained with cells transfected with wild-type CAS or CAS-SD without the gst tag (data not shown). (*A*, *middle*) Phosphotyrosine immunoblot of gstCAS-WT and gstCAS-SD immunoprecipitated from COS cells treated as for the migration assay above and attached to the culture dish. (*A*, *bottom*) The blot was reprobed with anti-gst antibodies to confirm equal loading of gstCAS-WT and gstCAS-SD. (*B*) Serum-starved FG-M cells were allowed to migrate on vitronectin- or collagen-coated Transwell membranes after being transiently transfected with either the empty expression vector or the vector containing CAS-SD along with the β-galactosidase reporter construct as described in the Materials and Methods. Migration was enumerated as described in *A* above. The results shown are representative of at least three independent experiments.

cells and that CAS-SD serves as a dominant-negative regulator of cell migration.

To establish whether the molecular interaction between CAS and Crk within these migratory cells depended on the CAS-SD domain, Crk was immunoprecipitated from an aliquot of the cells used for the migration experiments above. This immunoprecipitated material was then analyzed for the presence of tyrosine-phosphorylated CAS. As shown in Fig. 5 *B*, wild-type CAS, but not the CAS-SD, coprecipitated with Crk. As expected, the wild-type CAS, but not the CAS-SD, was highly tyrosine phosphorylated (data not shown). These same results were obtained when CAS was immunoprecipitated and probed for Crk association (Fig. 5 *C*). Endogenous Crk could also be coprecipitated with wild-type CAS but not the CAS-SD (data not shown).

To determine whether coupling of endogenous CAS/Crk proteins was associated with the migratory phenotype, FG or FG-M cells were either held in suspension or allowed to attach to vitronectin for 15 min and examined for changes in CAS/Crk binding. As shown in Fig. 5 *D*, FG-M cell adhesion to vitronectin resulted in significantly increased CAS/Crk coupling compared with FG cells. These findings reveal that both endogenous and exogenously expressed CAS and Crk interact with one another through tyrosine residues present in the substrate domain of CAS and that this interaction directly influences the migratory properties of these cells.

The CRK SH2 Domain Is Required for Binding to CAS and Induction of Cell Migration

To further establish the role of Crk/CAS binding in cell migration, cells were cotransfected with wild-type CAS and Crk with a point mutation in the SH2 domain (Crk-SH2) that prevents its binding to CAS (Matsuda et al., 1993; Tanaka et al., 1993). These cells were then tested for migration and their ability to assemble a CAS/Crk complex. Expression of this mutant Crk with wild-type CAS not only failed to induce cell migration but was also unable to couple to CAS (Fig. 6, *A* and *B*), indicating that Crk-SH2 served as dominant-negative in this case. Importantly, while these cells were deficient in migration, they showed no differences in their ability to attach or spread on various ECM proteins (data not shown). Together, these results support the notion that Crk binding to CAS is critical for the migratory properties of these cells and that the substrate domain of CAS as well as the SH2 domain of Crk contribute to this response.

Cell Migration Requires the NH_2-terminal SH3 Domain of Crk

Crk-dependent signaling events likely depend on the ability of this protein to couple via its two SH3 domains to one or more downstream effector molecules (Matsuda et al., 1994; Hasegawa et al., 1996). Therefore, we investigated the role of these SH3 domains in CAS/Crk migration. We first investigated the role of the NH_2-terminal SH3 domain in the cell migration response by expressing a Crk construct (Crk-SH3N) with a point mutation in this portion of the molecule, rendering it incapable of binding to effector molecules (Matsuda et al., 1994). Cells were transfected with wild-type CAS and either Crk or Crk-SH3N. While Crk-SH3N readily bound to wild-type CAS protein in these cells (Fig. 7, *bottom*), it did not promote cell migration, whereas the wild-type form of Crk did (Fig. 7, *top*). In contrast, expression of Crk with its COOH-terminal SH3 domain truncated readily bound to wild-type CAS and promoted migration that was comparable to that of cells

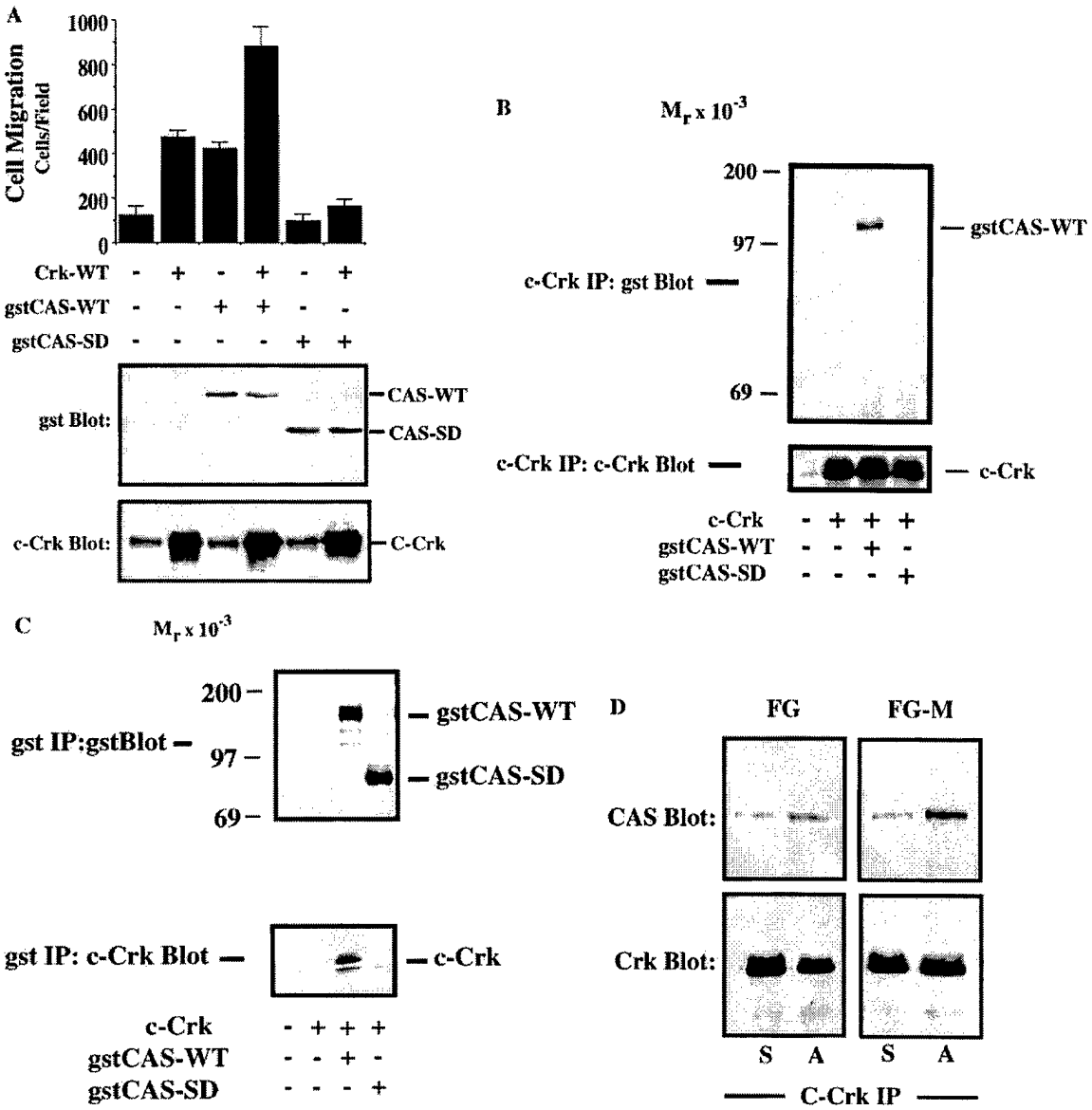

Figure 5. CAS and Crk promote cell migration. (*A*) Serum-starved COS cells were allowed to migrate for 3 h on vitronectin-coated membranes (*top*) or lysed on the culture dish and analyzed for expression of CAS (*middle*) and Crk (*bottom*) by immunoblotting after transfection with either the empty expression vector or the expression vector containing wild-type Crk (*Crk-WT*) and/or gstCAS-WT or gstCAS-SD. The number of transfected migratory cells that coexpressed β-galactosidase were enumerated by counting cells on the underside of the membrane as described in Materials and Methods. Each bar represents the mean ± SD of triplicate migration wells of one of three representative experiments. (*B*) Crk immunoprecipitated from detergent lysates prepared from COS cells transfected as for the migration assay above were analyzed by blotting with anti-gst (*top*) or anti-Crk antibodies (*bottom*). (*C*) gstCAS-WT or gstCAS-SD immunoprecipitated from detergent lysates prepared from COS cells treated as described in *B* was analyzed by immunoblotting with anti-gst (*top*) or anti-Crk antibodies (*bottom*). (*D*) Crk immunoprecipitated from detergent lysates prepared from FG or FG-M cells either held in suspension (*S*) or allowed to attach (*A*) to vitronectin for 15 min were analyzed by immunoblotting with anti-CAS (*top*) or anti-Crk antibodies (*bottom*). The results shown are representative of at least three independent experiments.

expressing wild-type CAS and Crk (data not shown). These findings reveal that the NH_2-terminal, but not the COOH-terminal, SH3 domain of Crk is critical for cell migration. Therefore, the commitment to migrate not only involves assembly of a CAS/Crk complex but depends on the NH_2-terminal SH3 domain of Crk, which is known to bind various effector molecules and promote downstream signaling events.

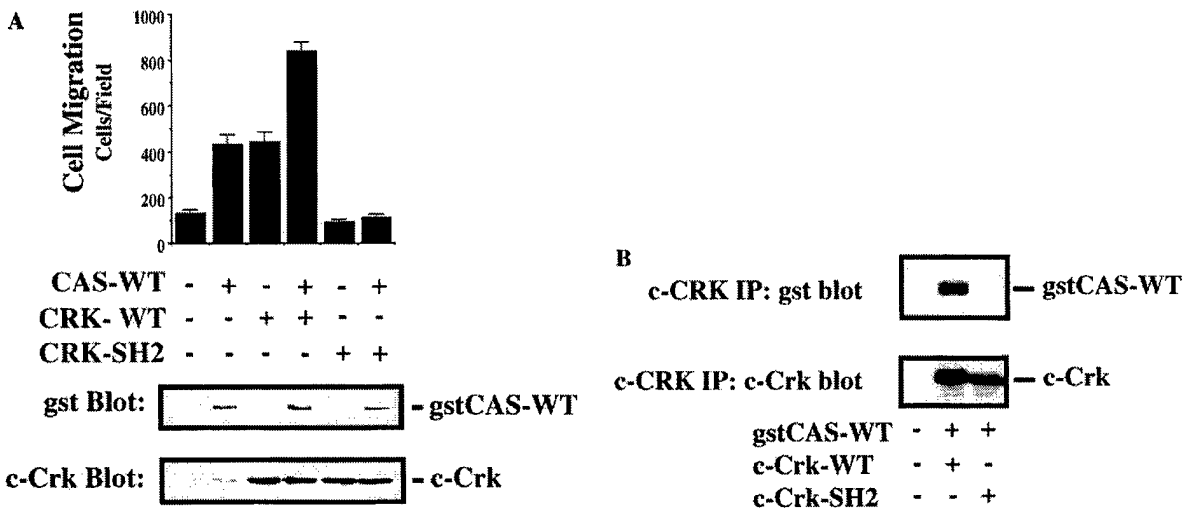

Figure 6. Crk binding to CAS is required for cell migration. (*A*) Serum- starved COS cells were allowed to migrate for 3 h on vitronectin-coated membranes (*top*) or lysed on the culture dish and analyzed for expression of Crk and CAS by immunoblotting (*middle* and *bottom*) after transfection with either empty expression vector or the expression vector containing gstCAS-WT and either Crk-WT or CRK with a mutated SH2 domain (*Crk-SH2*). The number of transfected cells migrating were enumerated by counting β-galactosidase–positive cells on the underside of the membrane. Each bar represents the mean ± SD of triplicate migration wells of one of three representative experiments. (*B*) Crk immunoprecipitated from detergent lysates prepared from COS cells transfected as described for the migration experiment in *A* and analyzed by immunoblotting with anti-gst (*top*) or anti-Crk antibodies (*bottom*). The results shown are representative of at least three independent experiments.

Insulin Promotes Migration in a CAS/Crk-dependent Manner

Previous work has established that cytokines or growth factors such as insulin are physiological activators of the molecular switch leading to cell migration/invasion. This is important during development, angiogenesis, and wound repair. In fact, various tumor cell lines stimulated with insulin or insulin-like growth factor not only show increased migration in vitro but gain the ability to metastasize in vivo (Klemke et al., 1994, 1997; Brooks et al., 1997). Serum-starved COS cells can be stimulated to migrate after treatment with various cytokines. Therefore, experiments were designed to investigate whether cytokine-stimulated cell migration was also dependent on CAS and Crk. To investigate this possibility, serum-starved COS cells expressing wild-type CAS or CAS-SD were allowed to migrate in the presence or absence of insulin. As shown in Fig. 8, expression of CAS-SD, but not wild-type CAS, in COS cells blocked insulin-induced cell migration without impacting the adhesion of these cells. Furthermore, expression of Crk, lacking a functional SH2 or an NH_2-terminal SH3 domain, also blocked this response (data not shown). Similar findings were obtained when cells were stimulated with IGF-1 and EGF (data not shown). Together, these findings demonstrate that CAS and Crk play a critical role in cytokine/growth factor–mediated cell migration.

Crk and CAS Localize to Membrane Ruffles in Migratory Cells

Targeting of signaling protein complexes to specific regions of the cell is important for the control of various cellular functions (Bar-Sagi et al., 1993). To investigate the intracellular localization of Crk and CAS, cells expressing wild-type Crk or CAS were allowed to migrate on vitronectin-coated coverslips for 2–4 h and then examined by immunofluorescent staining and confocal microscopy. As shown in Fig. 9 *A*, cells expressing either Crk or CAS had a classical motile phenotype with a leading lamellae containing membrane ruffles rich in F-actin. Interestingly, both Crk and CAS proteins were localized to these membrane ruffles (Fig. 9 *A*, *arrowhead*). Importantly, cells expressing wild-type CAS together with Crk showed a similar motile phenotype and colocalization of these proteins in membrane ruffles (data not shown). In contrast, cells within the field of view not expressing the Crk or CAS cDNAs contained little or no membrane ruffling and appeared nonmotile (Fig. 9 *A*).

To establish the subcellular localization of endogenous Crk and CAS, serum-starved cells were treated with or without insulin and stained for the presence of Crk and CAS. Nonmigratory serum-starved cells showed prominent Crk staining of focal contacts associated with the end of actin filaments at the cell–substrate interface (Fig. 9 *B*). However, after insulin treatment cells showed prominent membrane ruffles that contained both Crk and CAS (Fig. 9, *B* and *C*). Within the insulin treated cell population, few focal contacts or stress fibers were observed, which is characteristic of motile cells (data not shown). Together, these findings demonstrate that both Crk and CAS localize to the membrane ruffles of migratory cells.

Rac but Not Ras Activity Is Required for Crk/CAS-induced Cell Migration

The small G proteins Ras and Rac have been associated with cell movement (Ridely et al., 1992; Takaishi et al.,

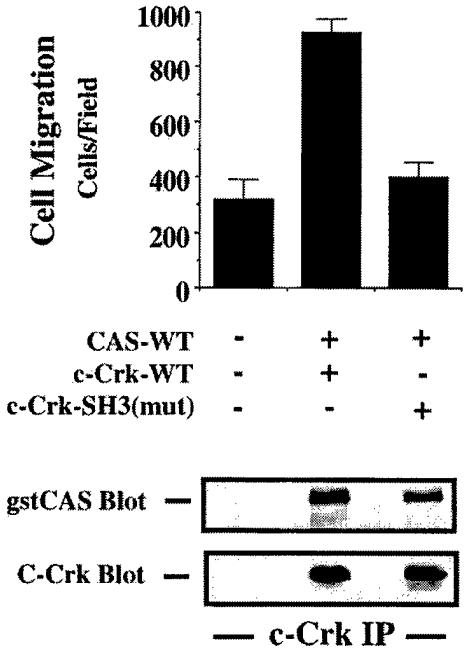

Figure 7. The NH_2-terminal SH3 domain of Crk is required for CAS/Crk-induced cell migration. (*Top*) Serum-starved COS cells were allowed to migrate for 3 h on vitronectin-coated membranes after transfection with either empty expression vector or the expression vector containing gstCAS-WT and either Crk-WT or CRK with a mutation in the NH_2-terminal SH3 domain (*c-Crk-SH3(mut)*). The number of transfected cells migrating were enumerated by counting β-galactosidase–positive cells on the underside of the membrane. Each bar represents the mean ± SD of triplicate migration wells of one of three representative experiments. (*Bottom*) Crk-WT or Crk-SH3N immunoprecipitated from detergent extracts from COS cells transfected as described for the migration experiment above and immunoblotted for gstCAS or Crk. The results shown are representative of at least three independent experiments.

1993; Michiels et al., 1995). In fact, membrane ruffling has been linked to the activation state of each of these small G proteins (Ridely et al., 1992). Therefore, we investigated the role of Rac and Ras in the Crk/CAS-induced migration. In these experiments, COS cells cotransfected with wild-type Crk and CAS together with either dominant-negative Rac (Rac−) or dominant-negative Ras (Ras−) were tested for their ability to migrate on ECM proteins. Expression of Rac− but not Ras− in these cells blocked Crk/CAS-induced cell migration (Fig. 10) and membrane ruffling (data not shown). In fact, expression of Ras− was greater than that of Rac− in these cells (Fig. 10), yet it still failed to suppress the Cas/Crk-mediated cell motility response. In addition, expression of a dominant-active form of Rac in these cells failed to promote migration, suggesting that Rac activity was not sufficient to facilitate this event (data not shown). Neither Rac nor Ras influenced the adhesive or spreading capacity of these cells, indicating expression of these constructs was not toxic (data not shown). Together these results indicate that Rac activity, but not Ras activity, is required for Crk/CAS-induced cell migration on the ECM.

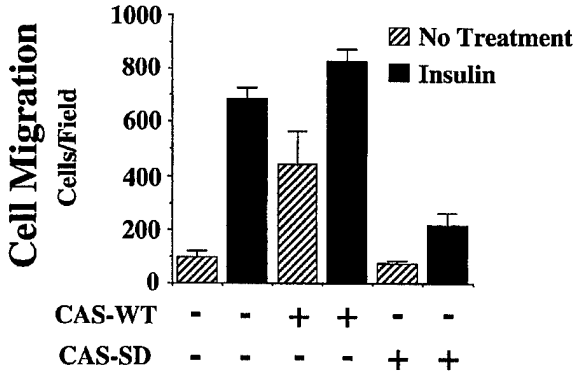

Figure 8. Requirement of CAS for cytokine-induced cell migration. Serum-starved COS cells were allowed to migrate for 3 h on vitronectin-coated membranes in the presence or absence of insulin after transfection with either empty expression vector or the vector containing gstCAS-WT or gstCAS-SD as described in Materials and Methods. The number of transfected cells migrating were enumerated by counting β-galactosidase–positive cells on the underside of the membrane. Each bar represents the mean ± SD of triplicate wells. Similar results were obtained after stimulation with IGF-1 and EGF (not shown).

Discussion

During wound repair and inflammation, cells gain the ability to migrate once they are stimulated with growth factors/cytokines. However, cell migration also depends on adhesive proteins present within the ECM (Clark et al., 1996; Palecek et al., 1997). In fact, both growth factor receptors and integrins promote intracellular signals that are associated with the migratory phenotype of cells (Ridley et al., 1992; Chen et al., 1994; Klemke et al., 1994, 1997; Brooks et al., 1997). However, little is known regarding how these signals activate the cells' migration machinery, thereby converting a stationary cell to a migratory cell.

While adhesion is necessary for cell motility, it is not sufficient. For example, FG cell attachment to collagen is permissive for migration, while attachment to vitronectin is not. This prompted us to examine whether FG cell attachment to collagen or vitronectin produced distinct signaling events. As we show in this study, the increased phosphorylation of CAS was associated with FG cell adhesion to collagen but not to vitronectin. In addition, selection of FG-M cells for their ability to spontaneously migrate on vitronectin resulted in a cell population that showed increased CAS phosphorylation and CAS/Crk coupling upon adhesion to vitronectin. It is this CAS/Crk complex that has been associated with downstream signaling events, yet little is known how these signals influence the biology of cells.

In this report, we demonstrate that assembly of the CAS/Crk adaptor protein complex serves as a molecular switch committing a cell to migrate on the extracellular matrix. This was investigated using cDNAs encoding alleles of wild-type Crk and CAS or cDNAs that had been mutationally altered in critical effector domains. Several lines of evidence are presented showing that CAS and Crk play a central role in cell migration. First, expression of wild-type CAS or Crk in cells was sufficient to induce cell movement without affecting cell adhesion on various ma-

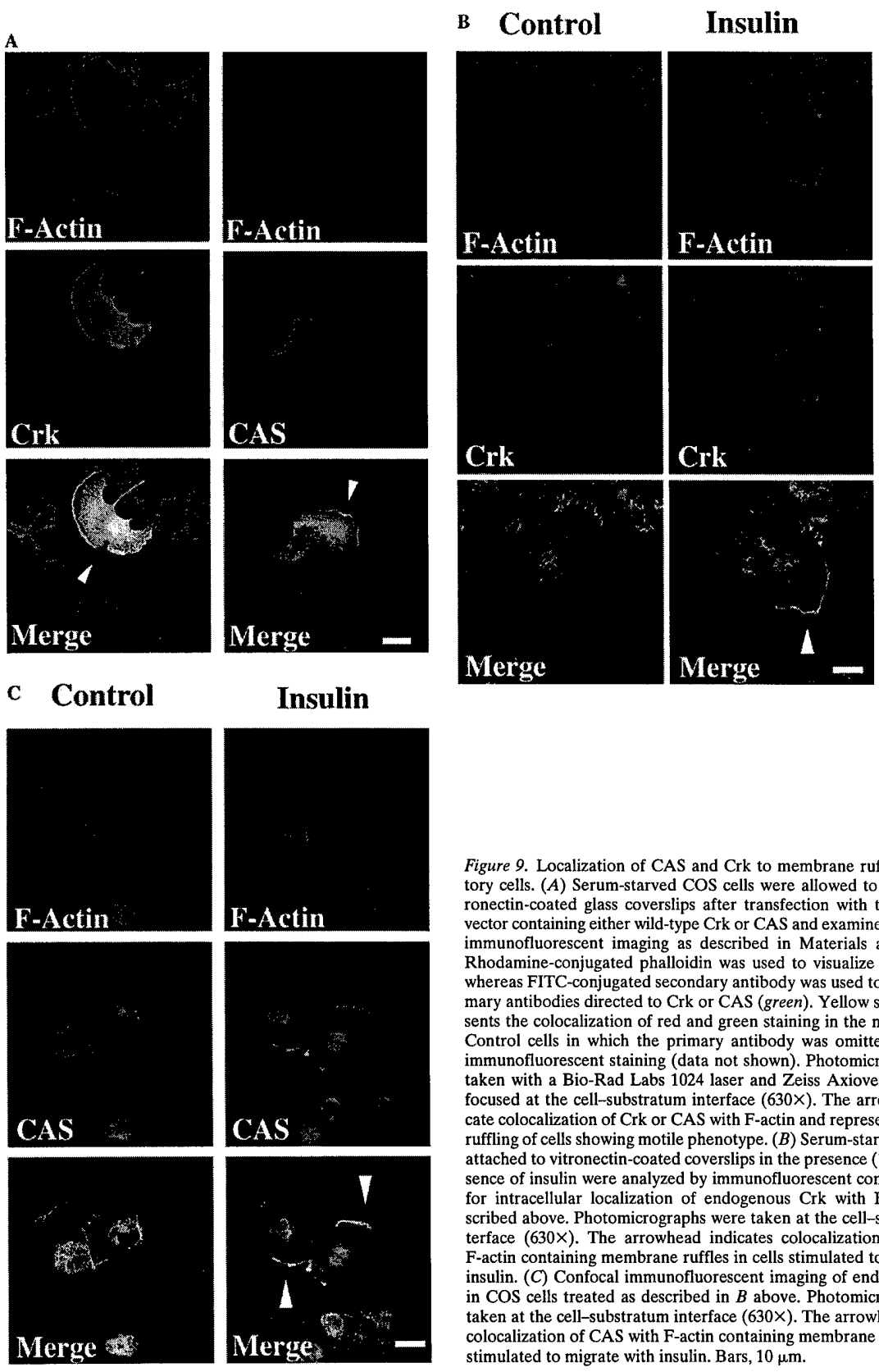

Figure 9. Localization of CAS and Crk to membrane ruffles in migratory cells. (*A*) Serum-starved COS cells were allowed to attach to vitronectin-coated glass coverslips after transfection with the expression vector containing either wild-type Crk or CAS and examined by confocal immunofluorescent imaging as described in Materials and Methods. Rhodamine-conjugated phalloidin was used to visualize F-actin (*red*), whereas FITC-conjugated secondary antibody was used to visualize primary antibodies directed to Crk or CAS (*green*). Yellow staining represents the colocalization of red and green staining in the merged image. Control cells in which the primary antibody was omitted showed no immunofluorescent staining (data not shown). Photomicrographs were taken with a Bio-Rad Labs 1024 laser and Zeiss Axiovert microscope focused at the cell–substratum interface (630×). The arrowheads indicate colocalization of Crk or CAS with F-actin and represent membrane ruffling of cells showing motile phenotype. (*B*) Serum-starved COS cells attached to vitronectin-coated coverslips in the presence (15 min) or absence of insulin were analyzed by immunofluorescent confocal imaging for intracellular localization of endogenous Crk with F-actin as described above. Photomicrographs were taken at the cell–substratum interface (630×). The arrowhead indicates colocalization of Crk with F-actin containing membrane ruffles in cells stimulated to migrate with insulin. (*C*) Confocal immunofluorescent imaging of endogenous CAS in COS cells treated as described in *B* above. Photomicrographs were taken at the cell–substratum interface (630×). The arrowheads indicate colocalization of CAS with F-actin containing membrane ruffles in cells stimulated to migrate with insulin. Bars, 10 μm.

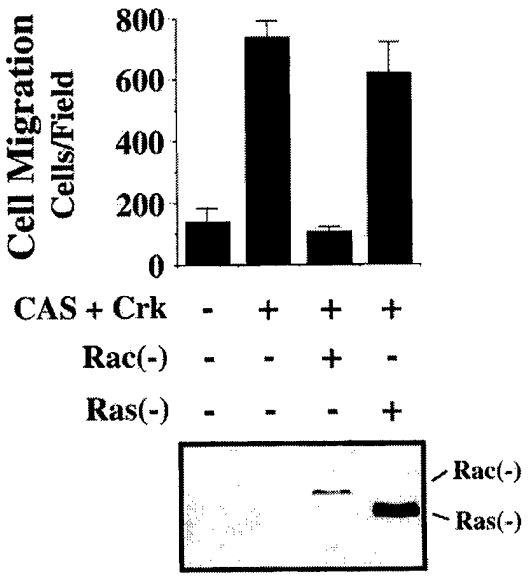

Figure 10. Requirement of Rac, but not Ras, for CAS/Crk-mediated cell migration. (*Top*) Serum-starved COS cells were allowed to migrate for 3 h on vitronectin-coated membranes after transfection with either the empty expression vector or the expression vector containing gstCAS-WT and Crk-WT along with dominant-negative Rac (−) or dominant-negative Ras (−) . The number of transfected cells migrating were enumerated by counting cells on the underside of the membrane as described above. Each bar represents the mean ± SD of triplicate migration wells of one of three representative experiments. (*Bottom*) An aliquot of those cells used for the migration assay above were lysed in detergent and analyzed for myc-tagged Rac or Ras expression as described in Materials and Methods.

trix proteins. Second, this migration event requires the assembly of a CAS/Crk complex involving the binding of Crk via its SH2 domain to the substrate domain of CAS. Third, cell migration induced by known regulators of motility including insulin, EGF, and IGF-1 required functional Crk and CAS, which were driven to the membrane ruffles of migratory cells. Finally, the small G protein Rac, which is involved in regulating signaling events important for membrane ruffling and cell migration, is required for migration induced by Crk/CAS. Surprisingly, a dominant-negative form of Ras failed to block CAS/Crk-induced motility (Fig. 10), even though it blocked cytokine-mediated cell migration (data not shown). These data suggest that Rac and Ras promote somewhat distinct pathways of cell migration. In fact, evidence from our laboratory demonstrates that the Ras/Map kinase (Erk 1 and 2) promotes cell motility based on the ability of ERK to phosphorylate myosin light chain kinase (Klemke et al., 1997). While a dominant-negative form of myosin light chain kinase blocked Ras-induced motility (Klemke et al., 1997), it failed to inhibit CAS/Crk-induced migration. Furthermore, the MEK inhibitor PD98059 failed to block CAS/Crk-induced cell migration (Klemke, R., unpublished data). Therefore, it appears that CAS/Crk-dependent cell migration may represent a distinct pathway from that initiated by Ras/MAP kinase.

CAS phosphorylation has been linked to cell adhesion and depends on kinases such as c-Src and FAK (Harte et al., 1996; Vuori et al., 1996; Schlaepfer et al., 1997); however, its susceptibility to phosphatases plays a key role in its level of phosphorylation (Garton et al., 1996). Therefore, exogenous expression of CAS could serve as a competitive substrate for such phosphatases, thereby increasing the overall level of phosphorylated CAS in the cell. In fact, we observed that exogenous expression of wild-type CAS not only caused increased migration but also led to increased phosphorylation of endogenous as well as expressed CAS protein (Fig. 4). Likewise, exogenous expression of Crk is known to protect CAS from dephosphorylation by phosphatases (Birge et al., 1992), providing a second mechanism for enhancing the level of phosphorylated CAS in these cells (data not shown). Therefore, exogenous expression of either wild-type CAS or Crk would be expected to enhance the level of CAS phosphorylation, resulting in the generation of CAS/Crk complexes.

This study demonstrates that CAS without a substrate domain and Crk without a functional SH2 or NH_2-terminal SH3 domain serve as dominant-negative proteins specifically blocking cell migration without affecting attachment or spreading of these cells on vitronectin, collagen, or fibronectin. CAS-SD may block migration by preventing CAS/Crk complexes from localizing to the appropriate subcellular location, such as focal adhesion sites or membrane ruffles. Both wild-type and CAS-SD have been shown to localize to focal contacts in adherent cells, an event that required the SH3 domain of CAS (Nakamoto et al., 1997). Expression of Crk-SH2 in cells may block CAS-mediated migration by competing with endogenous Crk for important effector molecules that associate with the NH_2-terminal SH3 domain. That expression of Crk-SH3 (N) in cells also blocks CAS-mediated migration supports this notion. In fact, several signaling molecules are known to interact with the NH_2-terminal SH3 domain of CRK, including Abl kinase (Feller et al., 1994*a*), DOCK 180 (Hasegawa et al., 1996), GTP exchange proteins SOS and C3G (Matsuda et al., 1994; Feller et al., 1995), and the EGF receptor substrate protein Eps15 (Schumacher et al., 1995). Alternatively, this SH3 domain may also be involved in targeting the CAS/Crk complex to a specific cellular location (Bar-Sagi et al., 1993). To this end, Crk and CAS were found to colocalize in membrane ruffles of migratory cells (Fig. 9).

While cell adhesion events are required for cell migration, not all adherent cells move. In fact, cell migration often depends on signals generated from both growth factor and adhesion receptors. Our findings suggest that CAS/Crk coupling provides the adhesion-dependent component of this signaling cascade and, thereby, serves as a molecular switch promoting cell migration on the extracellular matrix. However, in transformed cells, CAS/Crk coupling may be constitutive. In fact, v-Crk, a viral oncogene, has been linked to cell transformation and the malignant phenotype of tumors (for review see Matsuda and Kurata, 1996). We provide evidence that CAS/Crk signaling may also influence tumor cell metastasis. Specifically, FG-M carcinoma cells selected for spontaneous migration on vitronectin not only showed enhanced CAS phosphorylation and CAS/Crk coupling upon adhesion to vitronectin in vitro but also had an increased metastatic capacity in vivo compared with parental cells that showed no adhesion-dependent CAS phosphorylation.

The data presented here provide a mechanism to link adhesion-dependent signaling events and the formation of

a CAS/Crk complex to the activation of a Rac-dependent cell migration response. The phosphorylation of CAS and its coupling to Crk serves as a fundamental component of this signaling cascade. These events may be critical for growth factor– or cytokine-induced cell migration associated with development, wound repair, and inflammation. However, in transformed cells, CAS/Crk complexes may form spontaneously, leading to invasion events critical for the dissemination of tumors.

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Regulation of Cell Contraction and Membrane Ruffling by Distinct Signals in Migratory Cells

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Abstract. Cell migration and wound contraction requires assembly of actin into a functional myosin motor unit capable of generating force. However, cell migration also involves formation of actin-containing membrane ruffles. Evidence is provided that actin-myosin assembly and membrane ruffling are regulated by distinct signaling pathways in the migratory cell. Interaction of cells with extracellular matrix proteins or cytokines promote cell migration through activation of the MAP kinases ERK1 and ERK2 as well as the molecular coupling of the adaptor proteins p130CAS and c-CrkII. ERK signaling is independent of CAS/Crk coupling and regulates myosin light chain phosphorylation leading to actin-myosin assembly during cell migration and cell-mediated contraction of a collagen matrix. In contrast, membrane ruffling, but not cell contraction, requires Rac GTPase activity and the formation of a CAS/Crk complex that functions in the context of the Rac activating protein DOCK180. Thus, during cell migration ERK and CAS/Crk coupling operate as components of distinct signaling pathways that control actin assembly into myosin motors and membrane ruffles, respectively.

Key words: adaptor proteins • cell migration • mitogen-activated protein kinase • myosin • signal transduction

During development, wound repair and inflammation cells migrate in response to growth factors/cytokines and adhesive proteins present in the extracellular matrix (ECM)[1] (Lauffenburger and Horwitz, 1996; Keely et al., 1998). In many cases, these events are mediated by cytokine and integrin receptors that transmit a cascade of signals important for regulation of the migration machinery (Lauffenburger and Horwitz, 1996; Aplin et al., 1998; Keely et al., 1998). Initiation of migration is characterized by the rapid reorganization of actin to the cell edge. This results in the protrusion of a leading lamellipodium with new adhesive contacts necessary for traction. Membrane ruffles are often found on the cell surface and at the advancing front of a lamellipodium. They are thought to serve as sites of actin polymerization, endocytosis, receptor tyrosine kinase signaling, and protease activity (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). However, for a cell to move, it must also organize actin into a functional actin-myosin motor unit capable of generating contractile force. This propels the cell forward and contributes to the release of adhesive contacts at the rear of the cell (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Myosin II is the best-characterized myosin isoform and is well known to promote contraction in non-muscle cells (Kolega et al., 1993; Jay et al., 1995). The ability of myosin II to associate with actin and mediate contraction are modulated by the phosphorylation of the regulatory light chain by the Ca^{2+}/calmodulin-dependent enzyme myosin light chain kinase (MLCK) as well as dephosphorylation by myosin phosphatase (Adelstein, 1983; Yoshioka et al., 1998).

While significant progress has been made in identifying biochemical signals involved in cell migration, the relationship of these events and how they impact the migration machinery of cells are not well defined. Given the complexity of cell motility, it is not surprising that multiple signals regulate this process. Indeed, PLCγ (Chen et al., 1994), FAK (Ilic et al., 1995; Parsons, 1996; Cary and Guan, 1998), c-Src (Rodier et al., 1995; Boyer et al., 1997; Fincham and Frame, 1998), PI3 kinase (Keely et al., 1997), MAP kinase (Anand-Apte et al., 1997; Klemke et al., 1997; Wei et al., 1998), as well as Tiam1 (Michiels et al., 1995; Sander et al., 1998) and the Rho family of small

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1. *Abbreviations used in this paper:* β-gal, beta galactosidase; BDM, butanedione monoxime; CAS, p130Crk-associated substrate protein; Crk, c-CrkII; Crk-SH2, Crk without a functional src-homology 2 domain; ECM, extracellular matrix; ERK, extracellular-regulated kinase; GFP, green fluorescent protein; MLC, myosin light chain; MLCK, MLC kinase; SH2, src-homology 2 domain; SH3, src-homology 3 domain.

GTPases have all been shown to modulate cell migration (Keely et al., 1997, 1998; Tapon and Hall, 1997).

Recently, the adaptor proteins p130CAS (CAS) and c-CrkII (Crk) have been shown to be involved in cell migration (Cary et al., 1998; Klemke et al., 1998). Adaptor proteins (Nck, Crk, Shc, Grb2) do not contain catalytic activity but consist primarily of src homology 2 (SH2) and 3 (SH3) domains that serve to assemble signal generating complexes and target them to discrete sites within the cell (Bar-Sagi et al., 1993). For example, activation of cytokine and integrin receptors promotes tyrosine phosphorylation of the substrate domain of CAS and its association with the SH2 domain of Crk (Vuori et al., 1996; Casamassima and Rozengurt, 1997, 1998; Ojaniemi and Vuori, 1997). Crk, in turn, binds though its SH3 domain to several downstream effector molecules including the small GTPase activating proteins DOCK180, C3G, and SOS (Matsuda et al., 1994; Feller et al., 1995; Hasegawa et al., 1996). While the coupling of CAS to Crk can serve as a "molecular switch" leading to cell migration and metastasis (Klemke et al., 1998), it is not yet known how these proteins impact the migration machinery of cells. That CAS and Crk localize to focal contacts as well as membrane ruffles suggest this protein complex may regulate these processes in migratory cells. However, CAS and Crk may also facilitate cell migration through its ability to couple to NCK and/or SOS which is known to facilitate activation of the Ras/ERK pathway (Schlaepfer et al., 1997). Activated ERK can modulate integrin affinity and phosphorylate myosin light chain kinase leading to increased MLC phosphorylation (Hughes et al., 1997; Klemke et al., 1997). Both of these signaling events may be important for cell migration as MLC phosphorylation facilitates contraction and remodeling of adhesive structures (Adelstein, 1983; Lauffenburger and Horwitz, 1996; Chrzanowska-Wodnicka and Burridge, 1996; Mitchison and Cramer, 1996; Rosenfeldt et al., 1998). Furthermore, cell contraction and integrin aggregation can lead to activation of FAK (Chrzanowska-Wodnicka and Burridge, 1996). Since FAK and/or c-src that associates with FAK can tyrosine phosphorylate CAS, this could also facilitate CAS/Crk complexes and Rac activation (Vuori et al., 1996; Tachibana et al., 1997). Thus, it is feasible that CAS/Crk and ERK may cooperate to promote both actin-myosin function and membrane ruffling in migratory cells. In fact, v-Crk has recently been shown to promote activation of Rho which can facilitate MLC phosphorylation and migration (Altun-Gultekin et al., 1998; Yoshioka et al., 1998). Alternatively, these events may not be coupled, but represent separate biochemical pathways capable of regulating these cellular processes.

In this report, we investigate whether CAS/Crk coupling and ERK activation are the same or parallel signaling pathways involved in the regulation of actin assembly into membrane ruffles as well as myosin motors. Evidence is provided that CAS/Crk coupling regulates the migration machinery by promoting membrane ruffling independent of ERK signaling. In contrast, ERK, but not CAS/Crk, controls MLC phosphorylation leading to actin/myosin-mediated cell contraction. These findings suggest that during cell migration CAS/Crk and ERK signaling operate as distinct biochemical pathways necessary for membrane ruffling and cell contraction, respectively.

Materials and Methods

Expression Vectors and Reagents

The expression plasmid pUCCAGGS containing either full-length DOCK180, myc-tagged c-CrkII, c-CrkII cDNA with a mutated amino-terminal SH3 domain (tryptophan 109 to cysteine), or Crk with a mutated SH2 domain (arginine 38 to valine) have been previously described (Matsuda et al., 1993, 1994; Tanaka et al., 1993; Kiyokawa et al., 1998b). The pEBG expression plasmid containing glutathione-*S*-transferase–tagged or untagged wild-type p130CAS or CAS with an in frame deletion of its substrate domain (CAS-SD, amino acids 213–514) have been previously described (Mayer et al., 1995). Myc-tagged dominant negative Rac 1 (N17) and mutationally activated MEK in pcDNA3 has been previously described (Minden et al., 1995; Klemke et al., 1997). PD98059 (2-[2'-amino-3'methoxyphenyl]-oxanaphthalen-4-one; Calbiochem-Novobiochem). Anti-myosin antibody that recognizes the myosin IIB isoform present in COS7 cells was a gift from Dr. Robert Adelstein (Molecular Cardiology, National Lung Heart and Lung Institute, National Institutes of Health, Bethesda, MD). The phosphoERK antibody was from Promega. The anti-Rac, DOCK180, and myc antibodies were from Santa Cruz Biotechnology. FG-C are human pancreatic carcinoma cells stably overexpressing c-CrkII as previously described (Klemke et al., 1998).

Transfection of COS-7 and Analysis of Cell Migration

Transient transfection of COS cells and Transwell migration assays were performed as previously described (Klemke et al., 1998). In brief, COS-7 cells were cotransfected with lipofectamine and expression vectors containing cDNAs encoding wild-type and/or mutant forms of MEK, Crk or CAS, Rac, DOCK180, along with a reporter construct encoding β-galactosidase (pCMV5-β-gal) or green fluorescent protein (pEGFP-C1; Clontech). Control cells were mock-transfected with the appropriate amount of the empty expression vectors along with the β-gal reporter. Cells were allowed to incorporate the cDNA constructs for 6–8 h, washed, and then allowed to incubate for 40 h which provides optimal transient expression in these cells. COS cells were then prepared for haptotaxis cell migration using X-gal as a substrate and analyzed for expression of specific proteins by immunoprecipitation and immunoblotting as previously described (Klemke et al., 1998). Transfection efficiency and cell adhesion of these cells to purified extracellular matrix proteins were monitored as described below. The migration of FG cells, metabolic labeling of transfected cells with [^{32}P]orthophosphate, immunoprecipitation of myosin light chains, SDS-PAGE, and autoradiography were performed as previously described (Klemke et al., 1997).

Cell Adhesion and Transfection Efficiency

Controls for transfection efficiency and cell adhesion to ECM proteins were performed as previously described (Klemke et al., 1998). In brief, an aliquot of cells from the migration experiments above was allowed to attach to culture dishes coated with purified ECM proteins. The dishes were washed and adherent cells transfected with the β-gal reporter gene were detected using X-gal as a substrate according to the manufacturer's recommendation (Promega). In typical transfection experiments with these cells, we obtain 70–75% efficiency, as determined by counting the number of β-gal positive cells relative to the total number of cells attached per field (200×). It is important to note that in an individual experiment, transfection efficiently varies <10%. The efficiency and adhesion control assures that changes observed in cell migration is not simply the result of differences in transfection efficiency or expression of the β-gal reporter gene or differences in the ability of transfected cells to attach to the ECM.

Laser Confocal Imaging of Membrane Ruffles, Actin, and Myosin IIB

COS-7 cells were cultured on glass coverslips and then transfected as described above along with a reporter construct encoding either GFP or β-gal. Cells were serum-starved for 24 h then treated with or without insulin (10 μg/ml) or IGF-1 (20 ng/ml) for 15 min. In some cases, MEK or mock-transfected cells were exposed to 50 μM PD98059 for 2 h before being treated with insulin or IGF-1 as described above. To visualize F-actin containing membrane ruffles, cells were rinsed in PBS then fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained with rhodamine-conjugated phalloidin. Cells with prominent actin-rich

membrane ruffles were quantified blindly by two independent investigators and represents the average number of transfected cells (i.e., GFP cells) with ruffles of eight high powered fields (400×). To observe F-actin filaments and myosin IIB colocalization in cells, we used a procedure that preserves the association of these proteins in whole cells. In brief, cells were extracted with 0.1% Triton X-100 detergent, fixed in 4% paraformaldehyde, then stained with rhodamine-conjugate phalloidin and rabbit anti-myosin IIB, followed by FITC-conjugated secondary antibodies as previously described (Cramer and Mitchison, 1995). In some cases, cells transfected with the β-gal reporter construct were detected by staining with mouse anti-β-gal and donkey anti-Cy5-conjugated secondary antibodies along with three color cell fluorescence and a laser confocal microscope (Bio-Rad 1024 and a Zeiss axiovert microscope). Myosin content per cell was obtained by determining the fluorescence intensity (sum of total green pixels × intensity) in the green/FITC channel per cell area (μm^2) using Adobe Photoshop and IPLab Spectrum P computer software.

Cell Contraction of Collagen Gels

Cell contraction of a three-dimensional collagen matrix was performed as previously described (Rosenfeldt et al., 1998). In brief, 1×10^6 cells/ml collagen were cultured for 2 d in DME containing 10% FBS. Cells were serum-starved for 4 h then exposed for 60 min to serum-free culture media containing PD98059 (50 μM), M7 (1 μM; [(5-iodonaphthalene-1-sulfonyl)homopiperazine, HCL; Calbiochem-Novabiochem], or BDM (10 mM; butanedione monoxime; Sigma Chemical Co.). To initiate contraction, collagen gels were mechanically released from the culture dish in the continued presence of the inhibitors. The change in diameter in millimeters was measured with a ruler at various times after release. In some cases, cells were washed to remove the inhibitors then cultured an additional 24 h in drug-free media before initiation of contraction as described above.

Results

CAS/Crk Coupling and ERK Activation Represent Distinct Signals Necessary for Cell Migration

Recently, we reported that CAS/Crk coupling as well as ERK activation facilitate cell migration (Klemke et al., 1997, 1998). Each of these events were shown to be required for cell migration, yet it remains unclear whether these signals represent the same or parallel pathways involved in regulation of this process. To address this issue, cells were transfected with CAS lacking its substrate domain (CAS-SD) or Crk with a mutated SH2 domain (CRK-SH2), either of which are capable of preventing CAS/Crk coupling and downstream signaling events (Feller et al., 1994; Matsuda et al., 1994; Klemke et al., 1998). Cells containing these cDNAs were treated with the cytokine insulin and examined for migration as well as ERK activity. Expression of either CAS-SD or Crk-SH2 blocked insulin-induced cell migration, yet had no effect on ERK activity in these cells (Fig. 1 A). Similar findings were observed in cells expressing Crk with a mutated amino-terminal SH3 domain that retains its ability to couple to CAS, but is unable to link to downstream effector molecules such as DOCK180 or C3G (data not shown; Matsuda et al., 1994; Kiyokawa et al., 1998a,b). These findings reveal that disruption of CAS/Crk coupling or its binding to downstream effectors can suppress cell migration without influencing ERK activity.

To determine whether ERK signaling represents an independent pathway necessary for cell migration, cells were induced to migrate with insulin and then exposed to the compound PD98059 that blocks ERK kinase (MEK), and thereby prevents ERK activation (Dudley et al., 1995). These cells were then examined for cell migration, ERK

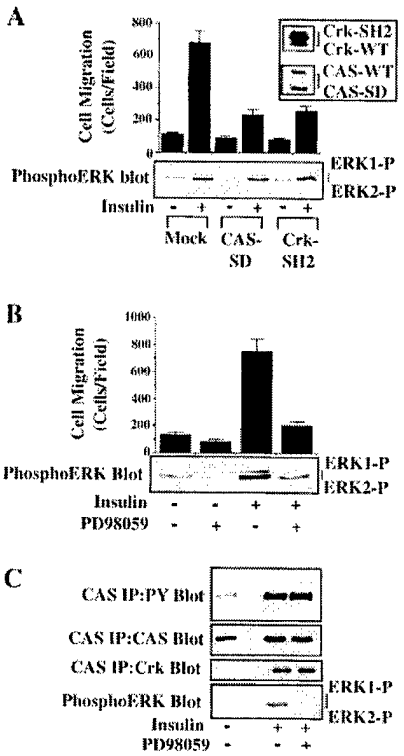

Figure 1. ERK activation and CAS/Crk coupling are separate signaling events necessary for cytokine-induced cell migration. (A) Serum-starved COS-7 cells were allowed to migrate for 3 h on vitronectin-coated membranes in the presence or absence of insulin (10 μg/ml) after transient transfection with a β-gal reporter construct, along with either the empty expression vector (Mock) or with expression vectors encoding gst-tagged CAS without its substrate domain (CAS-SD), or myc-tagged Crk with a mutated SH2 domain (Crk-SH2). CAS-SD and Crk-SH2 have been shown to prevent CAS/Crk coupling and downstream signals (Matsuda et al., 1993; Feller et al., 1994; Klemke et al., 1998). The number of transfected cells migrating were enumerated by counting cells on the underside of the membrane that coexpress the β-gal vector as described in Materials and Methods. An aliquot of cells treated as described for the migration experiment above was lysed in detergent and immunoblotted with antibodies to either the phosphorylated/activated form of ERK1/ERK2 (lower panel), Crk, or CAS (top right). Note that Crk-SH2 shows reduced mobility compared with wild-type endogenous Crk (Crk-WT) as the result of the molecular tag. (B) Serum-starved COS-7 cells were allowed to migrate in the presence or absence of the MEK inhibitor PD98059 (25 μM) with or without insulin (10 μg/ml). Cell migration and ERK1/ERK2 activity in these cells were determined as described above. Similar findings were obtained with fibronectin and collagen type I–coated membranes (data not shown). Each bar represents the mean ± SEM of at least three independent experiments. (C) Serum-starved COS-7 cells pretreated with or without the MEK inhibitor PD98059 (50 μM) for 2 h were exposed to insulin (10 μg/ml) for 5 min before being lysed in detergent. CAS was immunoprecipitated and then immunoblotted with antibodies to either phosphotyrosine, Crk, or CAS. The detergent lysates from these cells were also examined for changes in ERK1/ERK2 activity as described above. The result shown is representative from at least three independent experiments.

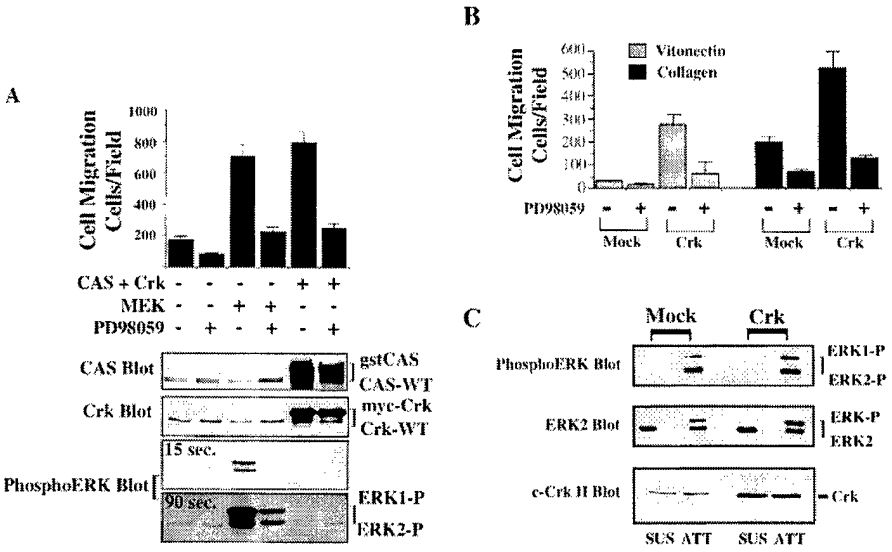

Figure 2. CAS/Crk-induced cell migration does not result from increased ERK activity. (A) Upper panel, serum-starved COS-7 cells were allowed to migrate for 3 h in the presence or absence of the MEK inhibitor PD98059 (25 μM) on vitronectin-coated membranes after transient transfection with a β-gal reporter construct, along with either the empty expression vector or with expression vectors encoding gst-tagged wild-type CAS and myc-tagged c-Crk or mutationally activated MEK. The number of transfected cells migrating was enumerated by counting cells on the underside of the membrane that coexpress the β-gal vector as described in Materials and Methods. Each bar represents the mean ± SEM of at least three independent experiments. Lower panels, total cell lysates prepared from cells treated as described for the migration experiments above were immunoblotted with antibodies that recognize the activated form of ERK1/ERK2 proteins, Crk, or CAS. Note that the gstCAS and myc-Crk proteins show reduced mobility compared with endogenous wild-type forms of these proteins as the result of the molecular tags. Immunoblots were exposed to film for 15 and 90 s after treatment with enhanced chemiluminescence reagents as described in Materials and Methods. (B) Serum-starved FG cells stably transfected with Crk or mock-transfected with the empty vector were allowed to migrate on vitronectin or collagen-coated membranes for 3 h in the presence or absence of PD98059 (25 μM) and quantified as described in Materials and Methods. Each bar represents the mean ± SEM of at least three independent experiments. (C) Cells treated as described for the migration experiment above were either held in suspension (SUS) or allowed to attach (ATT) to collagen-coated culture dishes for 30 min, then lysed in detergent and immunoblotted with either antibodies that specifically recognize the activated form of ERK1/ERK2 proteins (upper panel), ERK2 (middle panel), or Crk (lower panel). The upper band recognized by the ERK2 antibody represents the phosphorylated/activated form of this protein (ERK2-P) that has reduced mobility as a result of being phosphorylated. Similar results were obtained with ERK1 protein (data not shown).

activity, and formation of CAS/Crk complexes. While the MEK inhibitor prevented insulin-induced cell migration and ERK1/ERK2 activation, it did not impact CAS tyrosine phosphorylation or the formation of CAS/Crk complexes in these cells (Fig. 1, B and C). Similar findings were observed when cells were stimulated to migrate with either EGF or IGF-1 (data not shown). Thus, CAS/Crk coupling and ERK activation appear to represent components of distinct signaling pathways necessary for cytokine-induced cell migration.

To investigate directly whether formation of a CAS/Crk complex could activate ERK, serum-starved COS-7 cells were transiently transfected with vectors encoding CAS and Crk or mutationally activated MEK. These cells were then examined for ERK activity and migration. Expression of MEK in these cells promoted a four- to fivefold increase in cell migration and significantly increased ERK activity compared with mock-transfected control cells (Fig. 2 A). However, while CAS/Crk transfected cells showed a fourfold increase in cell migration, there was no change in ERK activity (Fig. 2 A). Similar findings were obtained in FG carcinoma cells stably transfected with c-Crk (FG-C). These cells also showed significantly enhanced migration, yet ERK activity was the same as control cells (Fig. 2, B and C). Together, these findings indicate that CAS/Crk-induced cell migration does not result from increased ERK activity. However, since ERK signaling appeared to be a separate event necessary for cell migration, we investigated whether ERK activity was also necessary for CAS/Crk-induced cell movement. To investigate this possibility, FG-C and COS-7 cells transfected with CAS and Crk were exposed to PD98059 and analyzed for their ability to migrate on ECM proteins. In this case, PD98059 blocked cell migration induced by CAS/Crk (Fig. 2, A and B) without affecting the formation of CAS/Crk complexes in these cells (data not shown). Conversely, MEK-induced cell migration was blocked by expression of CAS-SD or dominant negative RacN17 (Fig. 3, A and B) without impacting ERK activity (data not shown). Thus, CAS/Crk/Rac signaling and ERK activation appear to be separate biochemical pathways necessary for cell migration.

Membrane Ruffling Requires CAS/Crk and Rac, but Not ERK Activity

Activation of cell migration is characterized by the assembly of actin into membrane ruffles as well as cell contraction (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). To explain how the coupling of CAS/Crk and activation of ERK might influence the migration machinery, cells expressing CAS-SD were stimulated with insulin and then examined for actin-containing membrane

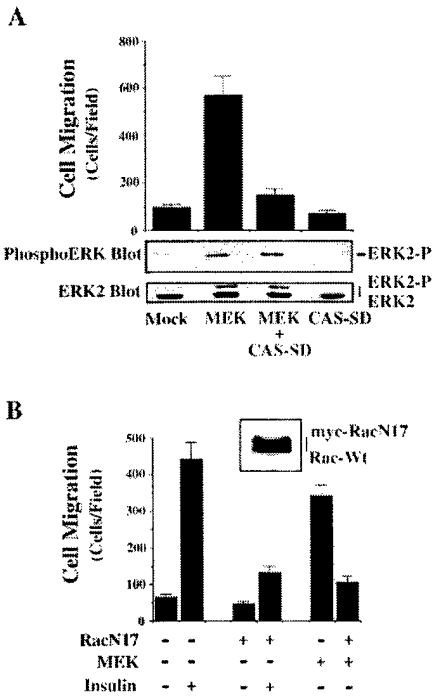

Figure 3. ERK-induced cell migration requires CAS/Crk and Rac activity. (A) Upper panel, serum-starved COS-7 cells were allowed to migrate for 3 h on vitronectin-coated membranes after transient transfection with a β-gal reporter construct, along with either the empty expression vector or with expression vectors encoding mutationally activated MEK, or MEK cotransfected with dominant negative CAS (CAS-SD). The number of transfected cells migrating were enumerated by counting cells on the underside of the membrane that coexpress the β-gal vector as described in Materials and Methods. Each bar represents the mean ± SEM of at least three independent experiments. Lower panels, cells treated as described for the migration experiment above were lysed in detergent and immunoblotted with antibodies to the activated form of ERK1/ERK2 (middle panel) or ERK2 (lower panel). The upper band recognized by the ERK2 antibody represents the phosphorylated/activated form of this protein (ERK2-P) that has reduced mobility as a result of being phosphorylated. Similar results were obtained with ERK1 protein (data not shown). (B) Serum-starved COS-7 cells were allowed to migrate for 3 h on vitronectin-coated membranes after transient transfection with a β-gal reporter construct, along with either the empty expression vector or with expression vectors encoding mutationally activated MEK, or MEK together with dominant negative myc-tagged Rac (RacN17) in the presence or absence of insulin (10 μg/ml) in the lower chamber. An aliquot of cells transfected with RacN17 and lysed and immunoblotted with an antibody to Rac is shown (top right). Note that RacN17 migrates slower as the result of the myc tag compared with endogenous wild-type Rac (Rac-Wt). Each bar represents the mean ± SEM of at least three independent experiments.

ruffles. Exposure of mock-transfected control cells to insulin induced prominent membrane ruffles rich in F-actin (Fig. 4 A). Approximately 18% of the control cell population showed membrane ruffling before stimulation with insulin and this was increased to 80% after cells were exposed to this cytokine (Fig. 4 B). Importantly, expression of CAS-SD in these cells completely blocked the insulin-induced membrane ruffling response (Fig. 4, A and B). As expected, cells within the field of view not transfected with CAS-SD showed prominent membrane ruffles (Fig. 4 A). Expression of Crk-SH2 in cells also blocked insulin-induced membrane ruffles (data not shown). Recently, it was reported that CAS/Crk coupling facilitates Rac activity which can promote membrane ruffling (Ridely et al., 1992; Kiyokawa et al., 1998a). Therefore, we determined whether CAS/Crk-induced membrane ruffles required Rac activity. Cells were transfected with CAS and Crk along with a dominant negative form of Rac (RacN17) and then examined for F-actin containing membrane ruffles. Expression of RacN17 in these cells blocked CAS/Crk-induced ruffles (Fig. 4 C). RacN17 also blocked cell migration without impacting ERK activity (data not shown). Thus, CAS/Crk coupling promotes membrane ruffles that depend on Rac, but not on ERK activity.

DOCK180 Potentiates CAS/Crk-mediated Cell Migration

Crk is known to bind to a number of downstream effector molecules via its SH3 domain, including c-Abl, SOS, C3G, Eps15, and DOCK180 (Matsuda et al., 1994; Feller et al., 1995; Hasegawa et al., 1996). Among these Crk-binding proteins, DOCK180 has been associated with Rac activation (Kiyokawa et al., 1998a). Moreover, in *Caenorhabditis elegans* and *Drosophila melanogaster*, the DOCK180 homologue ced-5 and mbc, respectively, control cell migration events associated with development (Erickson et al., 1997; Wu and Horvitz, 1998). Therefore, we were prompted to investigate the role of DOCK180 in cell migration. As shown in Fig. 4, D and E, expression of DOCK180 was able to potentiate CAS/Crk-induced cell migration in a Rac-dependent manner, yet it had no effect on ERK activity in these cells. Together these findings suggest that CAS/Crk in conjunction with DOCK180 can form a signaling module involved in Rac-mediated membrane ruffling and cell movement that is independent of ERK activation.

To investigate the role of ERK-dependent signaling in actin assembly, cells exposed to the cytokine insulin were treated in the presence or absence of the MEK inhibitor, PD98059. This inhibitor, which blocks cell migration, but not adhesion or spreading on collagen or vitronectin substrates (Fig. 1 B), failed to disrupt membrane ruffling in response to insulin (Fig. 4 A). In fact, 70% of cells exposed to PD98059 and insulin showed membrane ruffling, compared with 80% of cells exposed to insulin alone (Fig. 4 B). Together these findings indicate that CAS/Crk coupling is necessary for membrane ruffling, whereas ERK activity is not.

ERK Activity, but Not CAS/Crk Coupling, Is Necessary for Actin-myosin Assembly and Cell Contraction of a Three-dimensional Collagen Matrix

Cell migration also involves myosin light chain phosphorylation leading to actin-myosin association and cell contraction. While ERK can phosphorylate MLCK leading to increased MLC phosphorylation, it is not yet known if this event promotes assembly of a functional actin-myosin mo-

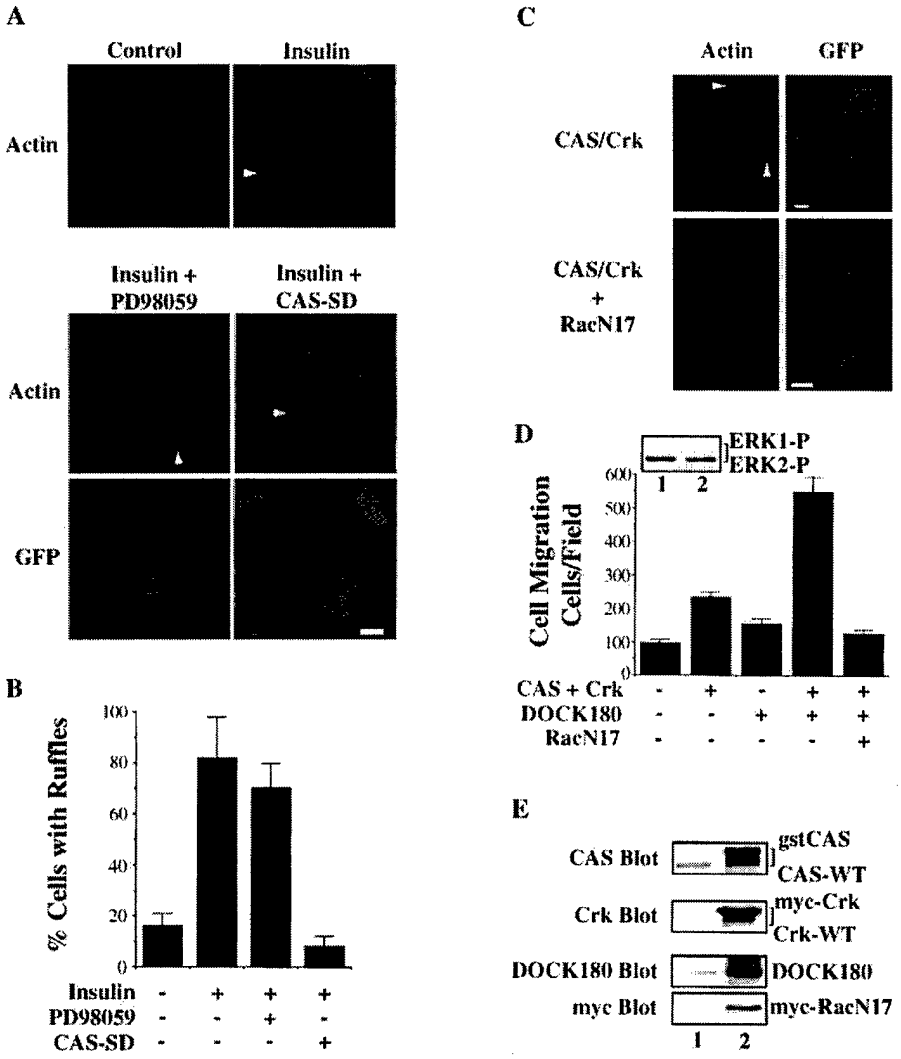

Figure 4. CAS/Crk association, but not ERK activation, is required for Rac-dependent membrane ruffling. (A) Serum-starved COS-7 cells in the presence or absence of insulin (10 μg/ml for 15 min) were stained with rhodamine-conjugated phalloidin, then analyzed by confocal imaging for F-actin (red) containing membrane ruffles after being transfected with either the empty vector (control) or the vector encoding dominant negative CAS (CAS-SD) along with a reporter vector encoding green fluorescent protein (GFP) to identify transfected cells. In some cases, control cells were pretreated for 2 h with 50 μM of PD98059 to inhibit ERK activity before being exposed to insulin as described above. Photomicrographs were taken with a Bio-Rad Labs 1024 laser and a Zeiss Axiovert microscope (400×). Arrowheads indicate cells with prominent F-actin membrane ruffles. (B) COS-7 cells treated as described above were scored for membrane ruffles as described in Materials and Methods. Results are expressed as the percentage of total transfected cells (i.e., green cells) that displayed prominent F-actin membrane ruffles and are the mean ± SEM of three separate experiments. (C) COS cells were transfected with expression vectors encoding CAS, Crk, and dominant negative RacN17, along with a reporter vector encoding GFP, then examined for actin membrane ruffles as describe above. Photomicrographs of CAS/Crk cells (400×) and RacN17 expressing cells (600×) were taken with a Bio-Rad Labs 1024 laser and a Zeiss Axiovert microscope. Arrowheads indicate cells with prominent F-actin membrane ruffles. (D) COS-7 cells transfected with either wild-type DOCK180, gst-tagged CAS and myc-tagged Crk, or CAS and Crk, together with DOCK180 and/or myc-tagged RacN17 were examined for cell migration as described above. An aliquot of cells transfected with CAS/Crk and DOCK180 (lane 2) or cells mock-transfected with the empty vectors (lane 1) as described for the migration experiment above were lysed in detergent and immunoblotted with antibodies to the phosphorylated/activated form of ERK1/ERK2 as described above (top left). Note that in these experiments cells were transfected with CAS/Crk vectors at DNA levels that give half-maximal migration. Each bar represents the mean ± SEM of at least three independent experiments. (E) An aliquot of cells treated as for the migration experiment above were lysed in detergent then immunoblotted with antibodies to CAS, Crk, DOCK180, or myc to detect myc-tagged RacN17. Lane 1, control cells transfected with the empty vectors. Lane 2, cells transfected with the vector containing the cDNA as indicated. Note that the gstCAS and mycCrk proteins show reduced mobility compared with endogenous wild-type forms of these proteins as the result of the molecular tag. Bars, 10 μm.

tor capable of generating force necessary for cell contraction (Klemke et al., 1998). This, and the fact that ERK did not influence membrane ruffling, yet appeared critical for cell migration, prompted us to examine its role in actin-myosin assembly and contractile function. We also investigated the role of CAS and Crk in this process since it is not known if these proteins regulate actin-myosin activity independent of their ability to organize actin into membrane ruffles. Cells were stimulated with insulin or transfected with mutationally activated MEK and examined for phosphorylation of MLC, which facilitates actin-myosin binding and motor activity (Adelstein, 1983). These cells were also examined for actin-myosin colocalization by immunofluorescent staining and laser confocal microscopy. Cells

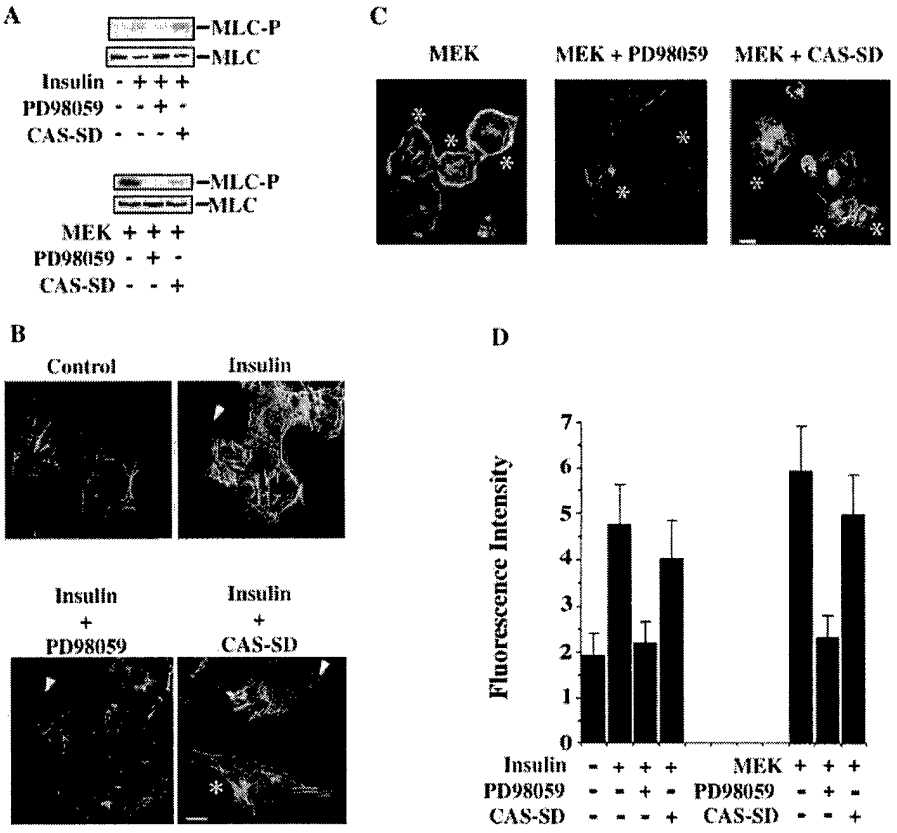

Figure 5. ERK, but not CAS/Crk signaling, promotes myosin light chain phosphorylation and actin-myosin colocalization. (A) Myosin light chains (MLC) immunoprecipitated with an anti-myosin IIB antibody from COS-7 cells metabolically labeled with [^{32}P]orthophosphate and treated with or without insulin (10 μg/ml) for 5 min as described in Materials and Methods. In some cases, ^{32}P-labeled cells were pretreated for 2 h with or without the MEK inhibitor PD98059 or transfected with mutationally activated MEK, and/or CAS-SD before immunoprecipitation of myosin IIB and SDS-PAGE as described above. MLC-P denotes phosphorylated light chain. MLC shows light chains stained with Coomassie before autoradiography to confirm that equal amounts of protein were precipitated in these experiments. (B) Serum-starved COS-7 cells either pretreated with the MEK inhibitor PD98059 (25 μM) or transfected with CAS-SD along with a β-gal reporter construct to identify transfected cells. Cells were stimulated with insulin (10 μg/ml) for 10 min, then exposed briefly to detergent to remove insoluble actin-myosin, fixed, and costained for actin and myosin IIB under conditions that preserve the association of these protein in cells as described in Materials and Methods (Cramer and Mitchison, 1995). Immunofluorescent laser confocal imaging was performed as described in Materials and Methods. Rhodamine-phalloidin (red) was used to visualize F-actin, whereas rabbit anti-myosin IIB and secondary FITC-conjugated goat anti-rabbit specific antibodies were used to visualize myosin (green). Yellow staining is the colocalization of red and green staining in the merged image. An asterisk indicates transfected cells as detected by immunostaining with a mouse anti-β-galactosidase antibody and a secondary antibody conjugated with Cy5 (blue, not shown). Arrowhead shows actin-containing membrane ruffles. (C) COS-7 cells transfected with mutationally activated MEK and/or CAS-SD along with a β-gal reporter construct to identify transfected cells and examined for actin-myosin association as described above. In some cases, MEK+ transfected cells were exposed to 25 μM PD98059 for 2 h to block MEK activation of ERK before staining for actin and myosin. Photomicrographs were taken with a Bio-Rad Labs 1024 laser and Zeiss Axiovert microscope (600×). An asterisk indicates transfected cells and arrow shows membrane ruffles. (D) Insoluble myosin content of COS-7 cells treated as described in B and C. Cells were stained with anti-myosin IIB and FITC-conjugated secondary antibodies and the total amount of green fluorescence intensity ($\times 10^6$) determined per cell area (μm^2) as described in Materials and Methods. Each bar represents the mean ± SEM of 30–40 cells of 6–8 different fields of three independent experiments. Bars: (B and C) 10 μm.

exposed to insulin or those expressing MEK+ showed increased phosphate incorporation into MLC compared with unstimulated or mock-transfected control cells (Fig. 5 A). Exposure to insulin or transfection with MEK also promoted increased actin-myosin colocalization compared with control cells (Fig. 5, B and C). In fact, the amount of insoluble myosin associated with these cells after extraction in detergent was increased by two- to threefold (Fig. 5 D). Importantly, PD98059 blocked insulin and MEK-induced MLC phosphorylation as well as actin-myosin colocalization, indicating that ERK activity was required for this response (Fig. 5, A–D). In contrast, transfection of cells with CAS-SD, which blocks membrane ruffling (Fig. 4), failed to block MLC phosphorylation and actin-myosin colocalization in response to insulin or expression of MEK+ (Fig. 5, A–D). Therefore, ERK activation selectively promotes MLC phosphorylation and actin-myosin colocalization, whereas CAS/Crk coupling facilitates membrane ruffling.

While cells require actin-myosin motor function for motility, these same events generate mechanical force necessary for wound contraction (Grinnell, 1994). Therefore, we investigated the role of ERK and CAS/Crk coupling in cell-mediated contraction of the extracellular matrix. Cells were transfected with CAS-SD or exposed to PD98059 and then examined for their ability to contract a three-dimensional collagen matrix. Exposure of cells to the MEK inhibitor significantly reduced the rate of cell con-

traction (Fig. 6 A). In fact, the time necessary to achieve half-maximal contraction was increased from 16 min in control cells to 36 min in cells exposed to the MEK inhibitor. Similar findings were obtained when cells were transfected with the ERK phosphatase MKP-2 which blocks ERK activity (data not shown). The time for half-maximal contraction was also significantly increased in cells exposed to the MLCK inhibitor M7 or in cells transfected with a dominant negative form of MLCK (MLCK−) (Fig. 6, A and B), which prevents ERK-induced cell migration and myosin light chain phosphorylation (Klemke et al., 1997). In this case, half-maximal contraction time was increased to 76 and 48 min in M7 and MLCK− cells, respectively, whereas control cells responded within 16 min. The difference in cell contraction between M7 and those transfected with MLCK− is likely due to the fact that ~75% of transfected cells express the MLCK− construct, whereas 100% of the cells are exposed to M7. In support of these results, cells exposed to BDM, a general inhibitor of myosin ATPase activity (Cramer and Mitchison, 1995), showed minimal contraction indicating that myosin plays a central role in this process (Fig. 6). In contrast, contraction was not altered in cells transfected with CAS-SD (Fig. 6 B) which blocks membrane ruffling, but not actin-myosin colocalization (Figs. 4 and 5). Similar findings were observed when cells were transfected with Crk-SH2 (data not shown). Importantly, the inhibition of cell contraction induced by these compounds was not due to nonspecific cell toxicity since their effect was reversed when they were removed from the culture media (Fig. 6 C). Together, these findings support the notion that ERK activation represents a distinct signaling pathway involved in the regulation of actin-myosin assembly and cell contraction, whereas CAS/Crk coupling represents an independent pathway that regulates actin membrane ruffling in migratory cells. However, while inhibition of ERK or MLCK activity decreased cell contraction, it did not completely block this event, suggesting that additional signals may exist to regulate MLC phosphorylation and actin-myosin contraction. Consistent with this possibility, Rho kinase is known to promote MLC phosphorylation independent of MLCK activity (Yoshioka et al., 1998).

Discussion

ERK activation and the molecular coupling of CAS and Crk are initiated upon cell adhesion to ECM proteins and/or exposure to various growth factors. Our findings that formation of a CAS/Crk complex and activation of the GTPase Rac are necessary for membrane ruffling, whereas ERK activity is involved in actin-myosin contraction, suggest that these signaling events regulate specific components of the migration/contraction machinery. During wound healing, the actin-myosin motor generates contractile force necessary for both cell migration as well as contraction of fibrin or collagen matrices. These cellular events appear to be independently regulated of CAS/Crk and Rac-associated events, while ERK regulates force generation and cell contraction.

Several lines of evidence suggest that CAS/Crk and ERK activation operate as components of separate signaling pathways necessary for cell migration. First, dominant negative forms of CAS and Crk that prevent CAS/Crk coupling blocked cytokine-induced cell migration without impacting ERK activation. Furthermore, inhibition of ERK activation prevented cytokine-induced cell migration without impacting CAS tyrosine phosphorylation or the formation of a CAS/Crk complex. Second, while formation of a CAS/Crk complex was sufficient to induce cell migration in serum-starved cells, it failed to promote ERK activation, indicating that this migration response was not the result of increased ERK activity. However, ERK activity was found to be a separate response necessary for CAS/Crk-induced cell migration since blocking endogenous ERK activity with PD98059 prevented CAS/Crk induced cell migration, but had no effect on the coupling of these proteins. In addition, we have observed that expression of mutationally activated MEK in cells, while sufficient to in-

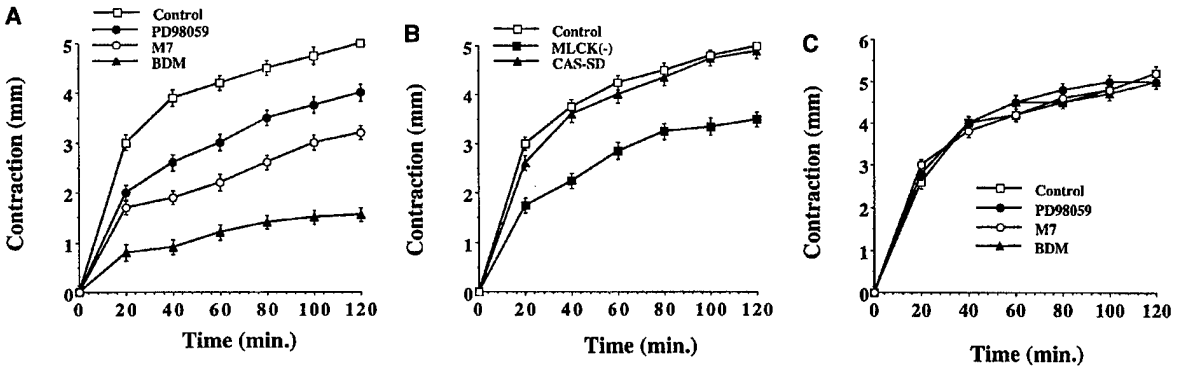

Figure 6. ERK activity, but not CAS/Crk association, is necessary for cell contraction of collagen gels. Three-dimensional collagen gels containing COS-7 cells were released from the culture dish and allowed to contract for various times as described in Materials and Methods. (A) COS-7 cells in collagen gels pretreated for 60 min with the MEK inhibitor PD98059 (50 μM), the MLCK inhibitor M7 (1 μM), or an inhibitor of myosin ATPase activity (BDM, 10 mM). (B) COS-7 cells transfected with either the empty expression vector (control) or the vector containing CAS without a substrate domain (CAS-SD) or a dominant negative form of MLCK (MLCK−) (Klemke et al., 1997). Contraction is presented as change in diameter (starting-final) measured in millimeters. (C) Cells treated as described in A were washed to remove inhibitors then cultured an additional 24 h before being allowed to contract the collagen matrix for various times as described in Materials and Methods. Each point represents the mean ± SEM of three independent experiments.

duce ERK activation and cell migration, did not impact CAS/Crk coupling (data not shown). That dominant negative forms of CAS and Crk were able to block MEK-induced cell migration, but not ERK activation, provided additional evidence that CAS/Crk and ERK are separate events required for cell migration. Finally, CAS/Crk and ERK represent distinct signals capable of regulating membrane ruffling and contraction in migratory cells. That Ras effector mutants deficient in their ability to facilitate ERK activity retain the capacity to promote membrane ruffling further support this notion (Joneson et al., 1996).

Our findings that CAS/Crk coupling was sufficient to induce membrane ruffles suggests that at least one important consequence of the molecular coupling of these proteins in cells is to facilitate Rac activation and/or its localization to the cell membrane. In fact, recent evidence indicates that CAS/Crk coupling in cells can potentiate Rac activity and cell spreading (Kiyokawa et al., 1998a). This was found to depend on the recently described Rac-activating protein DOCK180, which binds to the amino-terminal SH3 domain of Crk (Kiyokawa et al., 1998a). Our findings indicate that DOCK180 can potentiate CAS/Crk-induced cell migration, an event that depends on Rac activity. Together, these findings suggest that DOCK180 is an important downstream mediator in the CAS/Crk motility response. Interestingly, cellular expression of farnesylated, but not wild-type DOCK180, induced cell spreading and ruffling (Kiyokawa et al., 1998a), suggesting that DOCK180 requires localization to the cell surface to fully activate Rac and the associated changes in the actin cytoskeleton. During cell migration, CAS/Crk may serve to target DOCK180 to the membrane where it can interact with Rac. Indeed, CAS/Crk and Rac are known to localize to membrane ruffles of migratory cells (Tapon and Hall, 1997; Klemke et al., 1998). Alternatively, the association of DOCK180 with CAS/Crk complexes may regulate signaling from integrin adhesion receptors as recently suggested (Kiyokawa et al., 1998b). In either case, this appears to be independent of ERK signaling as cells expressing CAS/Crk/DOCK180 complexes showed enhanced cell migration without significant changes in ERK activity (Fig. 4 D). The ability of CAS/Crk complexes to couple to DOCK180 may have important implications for cell migration associated with development and cell metastasis. The DOCK180 homologues, mbc and ced-5, isolated from *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively, play a role in cell motility associated with the development of these organisms (Erickson et al., 1997; Wu and Horvitz, 1998). In contrast, CAS/Crk coupling may contribute to the migratory/invasive behavior of tumor cells through its ability to couple to the Rac and PI3 kinase signaling pathway (Keely et al., 1998). Indeed, carcinoma cells that showed increased invasive and metastatic potential in vivo were found to have increased CAS/Crk complexes compared with non-metastatic cells (Klemke et al., 1998).

While it is not yet clear how ERK is regulated in migratory cells, our findings indicate that CAS and Crk do not play a central role in this signaling cascade. Recent evidence suggests that several signals exist to regulate ERK activity independent of CAS and Crk (reviewed by Aplin et al., 1998; Schlaepfer et al., 1999). It is known that Src can phosphorylate FAK at tyrosine 925 leading to a Grb2/SOS association and direct ERK activation (Schlaepfer et al., 1994). Furthermore, the FAK-related tyrosine kinase Pyk2 directly couples integrin signals to ERK activation independent of CAS/Crk coupling (Blaukat et al., 1999). Protein kinase C and Grb2 binding to Shc provide additional pathways capable of regulating ERK activity in response to integrin events (Wary et al., 1996; Schlaepfer et al., 1998). Alternatively, CAS-dependent mechanisms may exist to link integrin and cytokine receptors to the RAS/ERK pathway. For example, Nck couples to SOS as well as tyrosine phosphorylated CAS. This could serve as an alternate pathway to facilitate a low level of ERK activity in some cells (Schlaepfer et al., 1997). However, it is not yet known if formation of a CAS/Nck complex is necessary for ERK activation or cell migration.

Assembly of an actin-myosin motor unit is critical for cell-mediated contraction of the ECM as well as cell movement, suggesting that these processes may be related. In fact, contraction of a collagen matrix involves a rapid smooth muscle–like contraction that is associated with increased ERK activity and myosin light chain phosphorylation (Grinnell, 1994; Rosenfeldt et al., 1998). Migratory cells also assemble actin-myosin motors and exert force on the ECM (Lauffenburger and Horwitz, 1996). This is thought to generate the force necessary for the rapid retraction of the tail region that is known to occur in migratory cells. Previous work has shown that ERK can directly phosphorylate and, thereby, activate MLCK leading to MLC phosphorylation (Klemke et al., 1997). In this report, we have extended these findings by showing that ERK activation can promote assembly of a functional actin-myosin motor unit capable of promoting cell contraction. Based on these findings, we propose that during cell migration ERK facilitates MLCK activity and MLC phosphorylation leading to the assembly of actin-myosin motors, an event necessary for cell contraction, but not membrane ruffling. On the other hand, CAS/Crk coupling independently regulates Rac activity and membrane ruffling in migratory cells. It is likely that additional signals operate to control cytoskeletal changes involved in cell movement. In fact, Rho modulates cell migration through its ability to inactivate myosin phosphatase leading to increased myosin light chain phosphorylation and cell contractility (Yoshioka et al., 1998). Furthermore, v-Crk can regulate Rho activity, suggesting that in some cells Crk may be able to facilitate myosin contractility (Altun-Glutekin et al., 1998). p21-activated kinase (Pak1) also regulates MLC phosphorylation and cell motility in fibroblasts (Sells et al., 1999), and Ras/ERK regulates integrin affinity and modulates adhesive contacts with the ECM, which is important for cell migration (Hughes et al., 1997).

Our findings that assembly of a CAS/Crk complex and Rac activation are necessary for membrane ruffling, whereas ERK activity facilitates actin-myosin contraction, indicate that these signals regulate specific components of the migration machinery. These findings provide molecular insight as to how cellular recognition of growth factors and adhesive proteins regulate the process of cell movement during development, wound healing, and inflammation, as well as tumor cell dissemination.

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Potentiation of Cell Migration by Adhesion-Dependent Cooperative Signals from the GTPase Rac and Raf Kinase*

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Running title: Rac in MAP kinase-mediated cell migration

Summary

The small GTPase Rac is thought to regulate cell movement by influencing actin cytoskeletal organization and membrane ruffling. However, cell migration also depends on the activation of mitogen activated protein kinase (MAPK)[1] which can regulate myosin motor function, an event critical for cell contraction. Evidence is provided that, during active cell adhesion to the extracellular matrix (ECM), Rac potentiates the MAPK pathway and influences cell migration by selectively synergizing with Raf kinase, but not Ras or MAPK kinase (MEK). In fact, the synergy between Rac and Raf kinase increases the chemotactic sensitivity of cells to epidermal growth factor (EGF) by 1000-fold. Therefore, the role of Rac in cell migration not only depends on its ability to regulate actin cytoskeletal organization but also on its capacity to potentiate chemokine activation of MAPK in a manner that depends on active cell adhesion to the ECM.

1. Abbreviations used are: ERK, extracellular signal-related kinase; MAPK, mitogen-activated protein kinase; MEK, MAP or ERK kinase; JNK, c-jun NH_2-terminal kinase; FBM, fibroblast basal medium; ECM, extracellular matrix; EGFR, epidermal growth factor receptor.

Introduction

Cell migration plays a critical role during development, wound repair, inflammation as well as tumor cell dissemination (1-2). These processes are regulated by chemokines and adhesion proteins which promote signaling events that influence cell migration by regulating actin organization and myosin motor function (3-6). The Rho family GTPases are known to affect the organization of the actin cytoskeleton in the migratory cell (7-8). Activation of Rho leads to the formation of actin stress fibers and focal adhesion assembly (9), while Cdc42 is involved in the induction of filopodia (7,10). The small GTPase Rac is known to promote actin cytoskeletal reorganization leading to membrane ruffling, and cell migration (11-12). Inactivation of Rac in Drosophila embryos lead to disruption of dorsal closure (13). In hematopoietic cells, T-lymphoma invasion and metastasis gene 1 (Tiam1), a known activator of Rac, promotes T cell invasion (14). In epithelial cells, active Rac and Cdc42 stimulate the motile behavior of T47D mammary carcinoma cells and this was associated with phosphatidyinositol 3-kinase activity (15). In some cases, Rho proteins can suppress the motile phenotype of cells. For example, Tiam1 and Rac inhibit hepatocyte growth factor (HGF)-induced scattering of epithelial Madin Darby canine kidney (MDCK) cells by increasing E-cadherin-mediated cell-cell adhesion (16). It was suggested that the intracellular localization of the GTPase signaling complex changed depending on which adhesive protein the cells were attached to and this dictated whether it stimulates cell-cell adhesion or cell migration (17). Therefore, it appears that Rho GTPase activities and their influence on cell biology, depend on the adhesive state of the cell.

Recent evidence indicates that the extracellular signal-related kinase (ERK) pathway contributes to cell migration in a manner independent of its ability to promote gene transcription or cell proliferation (18). Specifically, MAPK phosphorylates and thereby activates myosin light chain kinase (MLCK) which in turn phosphorylates myosin light chain (MLC) leading to cell migration in the absence of chemokines (18). In this case, phosphorylation of MLC by MLCK promotes actin/myosin assembly and ATPase activity

at the rear of migratory cells (6,19-20). However MAPK may influence motility by decreasing integrin-mediated adhesion (21-22). While Rac and MAPK appear to contribute to cell migration, it remains unclear how these molecules are regulated.

In this report, we investigated the role Rac plays in regulating the cell migration response. Evidence is provided that Rac not only promotes actin reorganization and membrane ruffling, but to our surprise, it increases the sensitivity of cells in response to EGF by 1000-fold. In this case, Rac is able to synergize with Raf kinase leading to increased MAPK activity in a manner dependent on active cell adhesion. These results demonstrate that Rac GTPase activity regulates cell migration in two ways, by inducing actin reorganization and in conjunction with raf kinase by potentiating MAP kinase activity.

Experimental Procedures

Antibodies and Reagents

Rabbit polyclonal antibodies to MAP kinases (ERK2 and JNK1), Ha-Ras, Raf1, MEK1 and EGFR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Flag M2 monoclonal antibody was purchased from Sigma Chemical Co. Anti-EGFR and anti-phosphotyrosine (4G10) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology, Inc. (Lake Placid, NY) respectively. Human recombinant EGF, PDGF-BB and insulin were obtained from Genzyme (Cambridge. MA). Anti active MAP kinase and PD 98059, a MEK inhibitor, were obtained from Promega (Madison,WI). A stock concentration (10 mM) of PD 98059 was prepared in DMSO and frozen at -70 ^{0}C. Myelin basic protein (MBP) was from Upstate Biotechnology, Inc. GST-c-Jun(1-169) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Rhodamine Phalloidin was from Molecular Probes (Eugene, OR).

Cell culture and Cell transfection

COS-7 and NIH3T3 cells were from ATCC (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1 mM glutamate. For transient transfection experiments, 100 mm dishes of cells 60-80% confluent were transfected using lipofectamine (GIBCO BRL) method according to the protocol of the manufacturer. 1 μg of pcDNA3 expression vector encoding mutationally active Rac1, H-Ras, Raf1, MEK1 or inactive Rac1 was used in keeping total DNA at 5 μg per dish. After 36 hr, COS-7 cells were placed in medium without serum for a further 18-24 hr prior to harvesting and assaying kinase activities and migration, and NIH3T3 cells were made quiescent by maintaining them in DMEM containing 0.5% serum for 16 hr. In typical transfection experiments we achieved a 80% expression efficiency providing a 10-

40 fold increase in the level of interest gene product compared to endogenous protein levels as determined by Western blotting (J. Leng, unpublished data). Cells used in migration assays, were co-transfected with 0.5 μg of a reporter construct encoding ß-galactosidase (pCMV-ß-galactosidase, Stratagene). X-gal was used as a ß-galactosidase substrate to visualize the transfected cells. Cells analyzed by immunofluorescent staining, were cotransfected with a green fluorescent protein (GFP) (pEGFP-C1, Clontech) as a cell transfection marker.

Cell Adhesion and Migration Assay

COS-7 cell migration assays were performed using modified Boyden chambers (6.5 mm diameter, 10 μm thickness, 8 μm pores, Transwell®; Costar, Cambridge, MA) containing polycarbonate membranes as previously described (23). The underside of the membrane of the upper chamber was coated with 10 μg/ml rat tail collagen type I (Upstate Biotechnology) or human fibronectin (Collaborative Biomedical Products) for 2 hours at 37 ^{0}C, rinsed once with PBS then placed into the lower chamber containing 500 μL migration buffer (fibroblast basal medium (FBM) with 0.5 % BSA, Clontics). Cells were removed from culture dishes with Versene (0.526 mM EDTA in PBS, Irvine Scientific, Santa Ana, CA), washed twice with migration buffer then resuspended in migration buffer (10^{6} cells/ml). 100,000 cells were added to the top of each migration chamber and allowed to migrate for 5 hours. In some cases, growth factors such as insulin or EGF were included in the lower chamber. Also, PD 98059 (25 μM) was used to pretreat cells for 1 hour before they were detached, and the cells were allowed to migrate in the presence of the inhibitor. Non-migratory cells on the upper membrane surface were removed with cotton swab and the migratory cells attached to the bottom of the membrane were stained with X-gal substrate. The number of X-gal positive migratory cells per membrane were counted with a Zeiss inverted microscope using a 10x objective in three randomly selected fields. Each determination represents the average of 3 individual wells. All values have had

background subtracted which represents cell migration on membranes coated on the bottom with BSA (1%).

Immunoprecipitation and Immunoblotting

Transfected COS-7 cells were rinsed twice with cold PBS, then lysed in RIPA buffer (100 mM Tris, 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% aprotinin, 2 mM PMSF, 10 μg/ml leupeptin, 5 mM EDTA, 1 mM sodium vanadate, and 50 mM NaF) for 30 min on ice. The lysates were clarified by centrifugation at 15000 g for 10 min at 4 ^{0}C, and protein concentration was determined using a Pierce Micro BCA protein assay kit (Pierce Chemical). EGFR from lysates containing 200 μg of total cellular protein were immunoprecipitated with protein G-bound anti-EGFR monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4 ^{0}C. Samples were subject to 8% SDS-PAGE and analyzed by immunoblots with anti-EGFR polyclonal antibody and antiphosphotyrosine monoclonal antibody. Lysates containing 2-10 μg of total cellular protein were also immunoblots with anti-ERK2 and anti-JNK1 polyclonal antibodies (Santa Cruz Biotech) for endogenous ERK2 and JNK1 respectively, anti-Ras, Raf, MEK antibodies for their expressions, and anti-Flag M2 monoclonal antibody (Sigma) for Rac1 expression and anti-ppERK polyclonal antibody (Promega) for active MAPK. Horseradish peroxidase conjugated goat anti-mouse or rabbit secondary antibodies and the enhanced chemiluminescence detection system (Amersham International) were used to visualize the blotted proteins of interests.

Protein Kinase Assays

The ERK2 and JNK1 activities in cell lysates were determined by an *in vitro* immuno-complex kinase assay. The ability of ERK to phosphorylate MBP was determined according to Boulton and Cobb (24). Briefly, 500 μg of protein from cell lysates were precleared with protein A-Sepharose for 4 hours in the cold and then incubated with protein

A-Sepharose coupled with anti-ERK antibodies (4 mg/100 ml stock bead suspension, Pierce) overnight in the cold. Immunoprecipitates were rinsed 3 times with RIPA and once with 50 mM Hepes (pH 8.0) containing 0.1 M NaCl before incubation with 100 µl of reaction mixture containing 10 µCi [^{32}P-] ATP, 10 mM $MgCl_2$, 20 µM ATP, 1 mM dithiothreitol, 1 mM benzamidine, 0.04 mg/ml MBP, and 25 mM Hepes pH 8.0, for 20 minutes at 30 ^{0}C. The reaction was stopped by adding 20 µl of boiling SDS sample buffer (6X). Phosphorylation of MBP was then analyzed by 15% SDS-PAGE, stained with coomassie blue, dried and exposed to imaging film overnight. Endogenous JNK activity was determined similarly by immuno-complex kinase assays after using an anti-JNK1 polyclonal antibody to immunoprecipitate JNK from cell lysate and its activity was measured using 2 µg of GST-c-Jun (1-169) as substrate (25).

Immunofluorescent staining

Transfected COS-7 cells grown on glass coverslips were serum-starved for 24 h and then treated with or without EGF (100 ng/ml) for 10 min. In some cases, cells were pretreated with PD 98059 (25 µM) for 30 min before EGF stimulation. Cell were fixed in 4% paraformaldehyde for 20 min at room temperature, permeablized with 0.2% Triton X-100 for 5 min, and stained with rhodamine-conjugated phalloidin (0.1 µg/ml) for 45 min. For all transfection cells, they were co-transfected with 0.5 µg of pEGFP as a reporter. Cell fluorescence was analyzed with a laser confocal microscope (Model 1024, Bio-Rad) with a 63x objective.

Results

To examine the effects of endogenous Rac on cell migration, COS-7 cells were transfected with Tiam1, a guanine nucleotide exchange factor specific for Rac that has been shown to promote T-lymphoma cell invasion into a fibroblast monolayer (14, 26). Cells expressing Tiam1 were allowed to migrate on a collagen-coated membrane in the absence of growth factors or chemokines. Tiam1 expressing cells showed a 3-4 fold increased haptotactic migration response when compared to mock transfected control cells (Figure 1A). This migration was blocked by expression of a mutationally inactive Rac (Rac1 N17) or by treatment of cells with an inhibitor of MEK (PD 98059) (27-28). These data indicate that cell migration induced with Tiam1 is dependent on both Rac and MAPK. Importantly, while Tiam1 expression enhanced cell migration it did not increase ERK activity in these cells as determined by an in vitro kinase assay using MBP as a substrate (data not shown). To further explore the roles of Rac and the MAPK pathway in migration, cells expressing active forms of Ras (H-Ras V12), Raf (Raf BXB) or MEK (MEK1EE) in the presence or absence of Rac1 N17 were allowed to migrate toward collagen. Active Ras, Raf or MEK promoted haptotaxis, which in each case, was blocked by co-expression of Rac1 N17 (Figure 1B).

Cells exposed to a chemokine gradient respond by moving toward the source of chemokine (29). To examine the role of Rac and MAPK in chemokine-directed cell migration, cells expressing Rac1 N17 or those exposed to PD 98059 were serum starved and allowed to migrate in response to the chemoattractants EGF or insulin. Both EGF and insulin induced cell migration that was completely blocked by expression of Rac1 N17 or exposure of cells to PD 98059 (Figure 1C). In addition, expression of a mutationally inactive form of MEK (MEK1AA) (28, 30), completely blocked EGF-induced cell migration confirming the results obtained with the MEK inhibitor PD 98059 (data not shown).

To investigate the general nature of these findings, we examined the role of Rac and MAPK in the migratory response of NIH3T3 fibroblasts to PDGF or expression of Tiam1. As shown in Figure 1D, like that observed for COS-7 cells, NIH3T3 cell migration induced by PDGF or Tiam1 expression of was blocked by expression of Rac1 N17 or treatment of cells with the MEK inhibitor PD98059. Together, these results demonstrate that both chemotactic and haptotactic cell migration depend on activation of the small GTPase Rac and the MAPK pathway.

The migratory property of cells depends on actin organization (9, 26). To examine the potential role of Rac and the MAPK pathway on actin organization, cells were treated in the presence or absence of EGF and transfected with mutationally active or inactive Rac. In some instances, cells were also exposed to the MEK inhibitor PD 98059. Cells were stained with Rhodamine-Phalloidin to visualize actin organization and membrane ruffling. Cells treated with EGF show intense membrane ruffling associated with the leading lamellipodia, while cells expressing active Rac alone, reveal extensive ruffling around the entire cell periphery (Figure 2). Cells exposed to EGF that also express mutationally inactive Rac (Rac1 N17) show no ruffling, while EGF-treated cells exposed to PD 98059 maintain active ruffling (Figure 2). These findings reveal that EGF, which induces motility, promotes actin reorganization and membrane ruffling in a Rac-dependent manner. Moreover, while suppression of MAPK activity blocks cells migration, it has no effect on membrane ruffling suggesting that MAPK and Rac differentially influence the actin cytoskeleton.

Directed cell migration during development, angiogenesis and inflammation depends on both cell adhesion to the ECM and on the ability of a cell to detect a chemokine gradient (31-34). To further evaluate the role of Rac in chemokine-dependent cell migration, cells were transfected with a mutationally active Rac (Rac1 L61) and allowed to migrate on a collagen substrate in response to a broad concentration range of EGF. To our surprise, cells expressing active Rac showed a 1000-fold increased sensitivity to EGF

compared to control cells. In this case, Rac potentiated cell migration toward 1 pg/ml EGF while control cells required >1 ng/ml to achieve this level of migration. Importantly, active Rac did not increase the overall level of migration but rather, enhanced the dose-response to the growth factor (Figure 3). COS-7 cell migration on collagen is mediated by $\alpha 2\beta 1$ integrin (18). EGF-induced cell migration on collagen was abolished by preincubating cells with P4C10 monoclonal antibody (anti-$\beta 1$ integrin) in the presence or absence of activated Rac (data not shown). Moreover, treatment of cells with EGF or expression of active Rac did not quantitatively or qualitatively influence the cells' adhesivity to a collagen substrate (data not shown). Together, these data demonstrate that Rac has the capacity to greatly potentiate the chemotactic effects of EGF enabling cells to respond to a minute dose of growth factor.

To move, cells must continually make and break adhesion contacts (35). Therefore, we examined the role of Rac in EGF-dependent ERK activation in cells that were either actively engaged in adhesion to simulate the migratory process or those that were pre-attached and spread for several hours on a collagen matrix. The ability of EGF to activate ERK was enhanced by Rac only in cells undergoing active adhesion (Figure 4A). Cells that had been pre-attached and spread on collagen for four hours, showed no such enhancement of ERK activity (Figure 4A). Importantly, cells expressing Rac in the absence of EGF showed no enhanced MAPK activity or migration regardless of their adhesive state. Therefore, EGF appears to cooperate with Rac in an adhesion-dependent manner to potentiate MAPK activity as well as chemotactic cell migration. These findings provide a link between cell adhesion events and the coupling of Rac activity to the MAPK-dependent cell migration machinery.

To investigate the mechanism by which Rac influences EGF-dependent cell migration, lysates from cells exposed to varying concentration of this chemokine in the presence or absence of Rac were examined for the level of EGF receptor (EGFR) phosphorylation. In this case, EGFR from cells expressing Rac1 L61 showed no increase

in tyrosine phosphorylation (Figure 4B) indicating that Rac impacts a downstream signaling event in the EGF-mediated cell migration response.

To begin to define the downstream mechanism(s) by which Rac influences the MAPK pathway and cell migration, cells were transfected with active Rac together with each component of the MAPK pathway. In this case, Rac was expressed in cells together with either a suboptimal dose (0.1 μg, DNA) or an optimal dose (1.0 μg, DNA) active Ras, Raf, or MEK. In the absence of exogenously expressed Rac, the optimal dose, but not the suboptimal dose, of Ras, Raf or MEK were sufficient to promote cell migration (Figure 5). In addition, cells containing Rac1 L61, alone or in conjunction with the suboptimal dose Ras or MEK showed no migration over background. However, cells expressing Rac in conjunction with the suboptimal dose Raf demonstrated a robust migration response (Figure 5). These data demonstrate that Rac can potentiate cell migration in a cooperative or synergistic manner with Raf kinase.

The fact that chemokines promote MAPK activity and induce cell migration prompted us to assess whether the cell migration response induced by the synergy between Raf and Rac might be associated with increased MAPK activity in these cells. Rac1 L61 was expressed in the presence or absence of 0.1 or 1.0 μg DNA encoding Raf BXB and lysates from these cells were assessed for MAPK activity using myelin basic protein (MBP) as a substrate. MAPK activity could not be induced when cells were exposed to 0.1 μg DNA (suboptimal dose) encoding Raf (Figure 6, middle panel). However, expression of 0.1 μg of Raf DNA together with Rac was able to induce MAPK activity to the same level as that observed with optimal level (1.0 μg DNA) of Raf alone (Figure 6, middle panel). While Rac synergized with Raf in promoting increased MAPK activity, no increase in Rac activation of endogenous c-jun N-terminal kinase (JNK) (36-37) was observed, as measured by phosphorylation of c-Jun (Figure 6, lower panel). Therefore, Rac and Raf function to preferentially enhance ERK activity.

To establish whether the migration response observed due to the cooperation of Rac and Raf requires MAPK activity, cells transfected with active Rac together with low dose (0.1 μg DNA) active Raf were incubated in the presence or absence of PD 98059 and allowed to migrate toward a collagen substrate. This MEK inhibitor completely abolished cell migration induced with Rac/Raf (Figure 7) indicating that this response requires MAPK activity. Therefore, we conclude that Rac and Raf can cooperate to activate MAPK and this is required for cell migration.

Discussion

Chemokines and adhesive components within the extracellular matrix initiate directed cell migration/invasion during development or in the adult organism, in response to injury or inflammatory events. Signals initiated by chemokine gradients and/or extracellular matrix proteins facilitate the conversion of cells from the stationary to the motile phenotype (38-39). Cell migration depends on reorganization of the actin cytoskeleton and development of cell polarity enabling a cell to make new adhesive contacts at its leading edge and break existing contacts at its trailing edge. This is associated with actin reorganization and in some cases active membrane ruffling at the cells' leading edge, an event mediated by the small GTPase Rac (9, 40) and contractile force at the rear of the cell which can be regulated by actin/myosin motor function (6, 41). Recent evidence demonstrates that myosin motor function can be regulated, in part, by MAPK which serves to phosphorylate and thereby activate MLCK (18). In fact, mutationally inactive MLCK or inhibitors of the MAPK pathway block MLC phosphorylation, and in so doing disrupt haptotaxis or chemotaxis (18). Alternatively, increased ERK activity may induce motility by reducing the adhesivity of integrin contacts with the matrix (21-22).

Evidence provided in this study indicates that Rac, commonly known to promote actin reorganization in motile cells, actually serves another primary function in the regulation of cell migration. During chemokine-induced migration activated Rac was able to increase the cells' sensitivity to EGF by >1,000-fold. This potentiation occurred downstream of the EGF receptor, since the presence of active Rac did not change the tyrosine phosphorylation profile of this receptor in response to varying amounts of EGF. Rather, it was associated with Rac's ability to enhance the activation of MAPK induced by EGF. Importantly, this increased MAPK activity by Rac could only be observed in cells that were actively attaching to their substrate, since stably preattached cells did not show this effect. In fact, active adhesion and deadhesion are ongoing processes in migratory

cells. Therefore, it is tempting to speculate that cell-associated proteases which can degrade ECM components and thereby release pre-attached cells from adhesive contacts may enhance chemotaxis, in part, by facilitating new adhesion events which we predict would enhance MAPK activity in a Rac-dependent manner. In addition to its ability to promote membrane ruffling, Rac can potentiate MAPK activity in response to EGF, an event critical for actin/myosin motor function (18, 42). We were able to separate these activities within the cell since, an inhibitor of MEK or a mutationally inactive form of this enzyme blocked cell migration without impacting the ability of Rac to promote membrane ruffling. In fact, immunofluorescent staining data presented documents that while MAPK is required for motility it does not influence membrane ruffling.

Rac's influence on MAPK activity and cell migration was based in part on its ability to synergize with Raf kinase. Thus, in cells with minimal stimulation of the Ras/MAPK pathway Rac appeared to amplify this response by potentiating Rafs ability to influence MAPK. Previous studies have indicated that Rac and Raf synergize and thereby enhance MAPK activity leading to increased cell proliferation (43-45). How this synergy occurs remains to be determined. However, it has been suggested that Rac can potentiate Raf's ability to activate MAPK through p21-activated kinases (PAKs), one of the downstream targets of Rac (42, 44). At first glance it may be surprising that Rac did not synergize with MEK, which is downstream of Raf. However, the selective synergistic effects between Rac and Raf may be related to the fact that both of these proteins are membrane coupled upon activation (17, 46-47), while MEK is not.

While some of our conclusions are based on experiments using exogenous expression of active or inactive Rac, we also observed that activation of endogenous Rac by the Rac guanine nucleotide exchange factor Tiam1 or chemokines such as EGF and insulin also promoted increased motility. Activation of endogenous Rac by Tiam1 leads to induction of motility while exogenous expression of active Rac in the absence of EGF fails to enhance motility. This apparent discrepancy can be explained by several possibilities. For

example, Tiam1 promotes the activity of endogenous Rac that leads to membrane ruffling at the cells leading edge, whereas, over expression of exogenous Rac1 L61 leads to membrane ruffling around the entire cell periphery (Figure 2). In addition, expression of exogenous Rac in cells may compete, in part, for endogenous Rac in terms of intracellular localization. Alternatively, Tiam1 might activate or indirectly potentiate the activity of other signaling molecules in the cell. In any event, Tiam1-induced motility or that induced by chemokines was blocked by either dominant negative Rac or inhibition of MAPK. The fact that inhibition of MEK blocked migration yet did not influence Rac-dependent membrane ruffling demonstrates that MAPK influences motility without impacting Rac or membrane ruffling. In contrast, Rac activity not only leads to membrane ruffling but, as shown here, it can potentiate MAPK activity in a cell expressing low levels of Raf kinase. This may explain how cells can be induced to migrate in vivo in response to relatively low levels of chemokine during the initial phases of angiogenesis or inflammation. Based on these findings we propose that cell motility depends on two signaling pathways, one involving Ras/MAPK activity leading to activation of actin/myosin motor function or and/or release of adhesion contacts, and a second that involves Rac-dependent membrane ruffling. Our results go on to document that Rac, once activated, can ultimately influence the MAPK pathway thereby enhancing the cell migratory response to chemotactic factors in an adhesion-dependent manner.

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Figure Legends

Fig. 1. Rac and MAPK activities are required for cell migration. (A) COS-7 cells were serum starved for 18 h and allowed to migrate for 5 h on collagen-coated transwell membranes after being transiently transfected with either the empty expression vector (pcDNA3) or active Tiam1 (C1199Tiam1) in the presence or absence of mutationally inactive Rac, Rac1 N17, as described in Experimental Procedures. In all cases, cells were cotransfected with a reporter construct encoding ß-galactosidase (pCMV-ß-galactosidase) for in situ ß-galactosidase staining with X-gal substrate. This facilitated enumeration of only those migratory cells that had been positively transfected. Transfection efficiency was routinely about 80%. In some case, cells were pretreated with 25 μM PD 98059 (MEK inhibitor) for 30 min prior to being allowed to migrate. Migratory cells (blue cells) on the underside of the migration chamber were counted using an Olympus (BX60) inverted microscope. Cells per high-powered (10 X) field were counted blindly by two observers. (B) COS-7 cells were transfected with active Ras (H-Ras V12), Raf (Raf BXB) or MEK (MEK1EE) in the presence or absence of Rac1 N17, and allowed to migrate toward collagen I substrate for 5 h as described in A. (C) Transfected COS-7 cells were pretreated with EGF (100 ng/ml) or Insulin (10 μg/ml) for 15 minutes on dishes. Cells were removed from culture dishes with Versene (0.526 mM EDTA in PBS), washed twice with migration buffer and resuspended in migration buffer. EGF or insulin was included in the lower compartment of the transwells. Cells were allowed to migrate for 5 h and cell migration was determined as described above. (D) NIH3T3 cells were serum starved for 16 h in 0.5% serum and allowed to migrate for 5 h on fibronectin-coated transwell membranes after being transiently transfected with either the empty expression vector (pcDNA3) or active Tiam1 (C1199Tiam1) in the presence or absence of mutationally inactive Rac (Rac1 N17), as described in Experimental Procedures. In some case, cells were also pretreated with 25 μM PD 98059 (MEK inhibitor) for 30 min prior to being

allowed to migrate. All data are presented as cell migration relative to control conditions and represent the mean ± SEM of three independent experiments.

Fig. 2. Rac, but not MAP kinase, mediates growth factor-induced membrane ruffling. COS-7 cells were transfected with empty vector or Rac mutants (active Rac (Rac1 L61) or inactive Rac (Rac1 N17)) together with a pEGFP reporter construct (Clonetics). After serum starvation for 24 h, cells were exposed to PD 98059 (25 μM) for 30 min before EGF treatment (100 ng/ml, 10 min). Actin polymerization was determined by staining with rhodamine-phalloidin as described in Experimental Procedures. GFP was used as a marker to visualize the transfected cells (data not shown). Photographs were taken with a Bio-Rad Labs 1024 laser and Zeiss Axiovert microscope (630 X). Arrowheads indicate the membrane ruffling areas.

Fig. 3. Rac potentiates EGF-induced cell migration. Serum starved COS-7 cells were allowed to migrate toward EGF for 5 h after they were transiently transfected with either the empty expression vector (open square) or the expression vector containing Rac1 L61 (close square). Data represents the cell migration response relative to controls and is determined from the mean ± SEM of four separate experiments performed independently.

Fig. 4. Rac potentiates EGF-induced ERK activation. Transfected COS-7 cells were serum starved for 24 hours and allowed to adhere to Collagen I coated dishes (10 μg/ml) in the presence of EGF as indicated for 60 min (attaching cells). Alternatively, preattached cells (4 h) were treated with EGF for 60 min as indicated. (A) Cell lysates were analyzed for the expression of Rac1 L61 and the endogenous level of ERK2 by immunoblots. The activity of MAPK was determined by kinase assay using MBP as a substrate. (B) The average MAPK activity (MBP) was determined for actively attaching cells treated with the indicated EGF concentrations. Each point represents the mean +/- SD

of three independent experiments. (C) EGFR was immunoprecipitated from cell lysates and its total level or phosphotyrosine content was examined as described in Experimental Procedures. Shown is a representative experiment from three independent experiments.

Fig. 5. Rac synergizes with Raf, but not with Ras or MEK, to promote cell migration. COS-7 cells were transfected with 1.0 μg DNA encoding active Rac (Rac1 L61), together with 0.1 μg or 1.0 μg DNA encoding active Ras (H-Ras V12) (A), Raf (Raf BXB) (B) or MEK (MEK1EE) (C). Relative levels of Ras, Raf or MEK expression were determined by immunoblot analysis. Cell migration data are expressed as the relative fold induction over controls from the mean ± SEM of three independent experiments.

Fig. 6. Rac synergizes with Raf to activate MAPK. COS-7 cells were transfected with 0.1 μg or 1.0 μg DNA encoding active Raf (Raf-1 BXB) and with 1 μg DNA encoding active Rac (Rac1 L61). Lysates from these cells were immunoblotted using anti-Flag M2 monoclonal antibody to measure the expression of exogenous Rac. ERK2 and JNK1 levels and their activities were determined as described in Experimental Procedures. Shown is a representative experiment from three independent experiments.

Fig. 7. Cell migration induced by Rac and Raf synergy is dependent on MAPK activity. COS-7 cells were transfected with 0.1 μg or 1.0 μg DNA encoding active Raf (Raf-1 BXB) and with 1 μg DNA encoding active Rac (Rac1 L61). Cell migration assays were performed in the presence or absence of PD 98059 (25 μM) as described in Experimental Procedures. Data are expressed as the mean ± SEM of four independent experiments.

001 - Chambers

Clinical targets for anti-metastatic therapy: Extravasation vs. tissue-specific growth

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Metastasis is responsible for most cancer deaths. Therapeutic strategies to prevent formation of metastases or to minimize their clinical consequences are thus needed. Understanding the biology of metastasis will assist in appropriate design and use of anti-metastatic therapeutics. It is crucial that individual steps in the metastatic process be defined, steps vulnerable to intervention be identified and steps where proposed molecular interventions contribute be determined. We have used in vivo videomicroscopy to quantify steps in metastasis in mice, and a "cell accounting" procedure to monitor survival of cells. These studies have provided new insights about the metastatic process. It was believed that most cancer cells are destroyed in the circulation and that few remaining cells could extravasate (escape from the circulation). We quantified proportions of melanoma cells, injected via mesentery vein to target liver, that survived and extravasated, formed micrometastases and developed into macrometastases. Nearly all cells survived and extravasated, with many then remaining dormant. Contributors to metastatic inefficiency included initiation of growth to form micrometastases, and continued growth of a subset of these. We also assessed steps at which the metalloproteinase inhibitor batimastat inhibited liver metastasis. Extravasation, survival of cells and micrometastasis formation were not inhibited. The major effect of batimastat in this model was to inhibit angiogenesis of liver metastasis. Together these studies suggest that post-extravasation growth may be an appropriate target for anti-metastatic therapeutics.

002 - Klemke

Regulation of Cell Migration by CAS/Crk Coupling

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Carcinoma cells selected for their ability to migrate in vitro showed enhanced invasive properties in vivo. Associated with this induction of migration was the anchorage-dependent phosphorylation of the adaptor protein p130 CAS and increased CAS/Crk binding. In fact, expression of CAS or its adaptor protein partner Crk was sufficient to promote cell migration and this depended on CAS tyrosine phosphorylation leading to an SH2-mediated complex with Crk. Cytokine-stimulated cell migration was blocked by CAS lacking the Crk binding site or Crk containing a mutant SH2 domain. This migration response was characterized by CAS/Crk localization to membrane ruffles and blocked by the dominant negative GTPase, Rac, but not Ras. Thus, CAS/Crk assembly serves as a "molecular switch" for the induction of cell migration and appears to contribute to the invasive property of tumors.

003 - Condeelis

EGF-receptor-stimulated Chemotaxis in Breast Adenocarcinoma Cells: Molecular Mechanisms Relating Protrusion, Adhesion and Protein Synthesis

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The Metastatic mammary adenocarcinoma cell line MTLn3 responds to EGF or TGF-α by rapidly extending lamellipods and changing the pattern of focal adhesion contacts with extracellular matrix. Non-metastatic clones from the same tumor do not exhibit these responses. Further study of MTLn3 cells demonstrates that the cells are capable of true chemotaxis, i.e. the ability to follow faithfully a micropipette filled with EGF. In addition, cells labeled with GFP form tumors in vivo that exhibit highly polarized locomotion consistent with chemotaxis. EGF induced protrusion activity does not require the formation of adhesive contacts but adhesion regulates the shape, size and stability of protrusions. The EGF induced protrusion contains a zone of intense actin polymerization within 0.3um of the plasma membrane. EGF stimulates the formation of this zone by increasing the number of filaments and barbed ends locally under the plasma membrane. Treatment of cells with antisense but not sense oligonucleotides against the severing protein cofilin blocks pseudopod extension in MTLn3 cells. Since the crosslinking of actin filaments allows polymerization to generate force for protrusion, the above results indicate that EGF-induced severing causes increased barbed-end dependent actin polymerization leading to lamellipod extension. EF1α is an F-actin crosslinker present in the leading edge.
EF1α is also a metastasis associated protein that is overexpressed in a variety of metastatic tumors. EF1α and mRNA moves rapidly to the leading edge of the protrusion during locomotion. The extent to which this occurs is inversely correlated with metastatic potential in these rat adenocarcinomas. EF1α crosslinks F-actin, regulates the rate constants of actin polymerization and may participate in targeting of mRNA to filaments near the polymerization zone. The relationship of actin polymerization, protrusion and protein synthesis in establishing and maintaining cell polarity of locomotion during metastasis will be discussed.
(support from DAMD17-9616129, GM25813, GM38511 and USAMRDC 2466).

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Nitric oxide induces dose-dependent changes in $[Ca^{2+}]_i$, morphology and migration of human neutrophils. Vesa M. Loitto, Tommy Sundqvist and Karl-Eric Magnusson. Dept. of Med. Microbiol., Link "ooping University, Link "ooping, Sweden

To assess the role of nitric oxide (NO) on neutrophil Ca^{2+}-signaling and migration, we added different concentrations (0.5-800 μM) of the NO-radical donor S-Nitroso-N-Acetylpenicillamine (SNAP) to adherent neutrophils. Neutrophils were isolated from peripheral blood, loaded with Fura-2 to assess changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) with ratio imaging microscopy in parallel to video imaging of cell morphology and migration. The NO-generation induced a rapid and persistent morphological hyperpolarization followed by migrational arrest suggesting NO-mediated repression of F-actin turn-over. Occasionally, the cells regained motility after ca 10 min. The inhibition of locomotion was usually preceded with a Ca^{2+}-transient. The SNAP-induced elevation of $[Ca^{2+}]_i$ was concentration-dependent with an optimal effect above 50, but below 500 μM. This effect was likely due to NO formation, since D-penicillamine, the other by-product of SNAP, did not affect $[Ca^{2+}]i$. Furthermore, increasing doses of SNAP (*200 μM) attenuated the response to the chemoattractant f-met-leu-phe (fMLP). Thus, neutrophils exposed to NO_i first express a polarized morphology, presumably through an initial increase in $[Ca^{2+}]_i$, and then inhibition of migrational activity, indicating an inability of the cells turn-over of newly formed microfilaments.

1682

IDENTIFICATION OF A FUNCTIONAL EPITOPE IN CD9 EXTRACELLULAR DOMAIN ASSOCIATED WITH HAPTOTACTIC MOTILITY ((JX Bao, JT Crossno, Jr., MM White, LK Jennings.)) Departments of Medicine & Biochemistry, The University of Tennessee, Memphis 38163.

The tetraspanin CD9 is a transmembrane cell surface protein involved in cell adhesion, motility and proliferation. In previous studies, we have established that fibronectin (Fn) is a ligand for CD9 and that a Fn-binding site is located in the second extracellular loop of CD9. In this study, CHO cell clones expressing CD9 containing deletions in second extracellular domain were analyzed for haptotactic motility. CD9 surface expression was confirmed by antibody staining and flow cytometry. We observed that CD9-transfected CHO cells have a five-fold increase in motility to Fn as compared to mock-transfected cells. CHO cells expressing the second extracellular loop deletion containing the Fn-binding site exhibited reduced Fn-mediated haptotactic motility. Cells expressing a deletion of amino acid residuals 152-192 containing the complete Fn-binding site demonstrated a 56% decrease in haptotactic motility over 3 hours and a 60% reduction in motility over 6 hours ($p<0.01$). Cells expressing a deletion in amino acid residuals 133-192 exhibited a 52% reduction in motility over 3 hours and 64% motility over 6 hours ($p<0.01$). In addition, cells transfected with CD9 deletion mutant containing a partial Fn-binding site (deletion in amino acid residuals 173-192) exhibited an equivalent haptotactic motility as compared to full length CD9 after 3 hours, but the motility was reduced to 45% over 6 hours. Peptide inhibition studies showed that CD9-mediated motility was competitively inhibited by a synthetic peptide homologous to the CD9 Fn-binding site sequence in a dose dependent manner. These studies support the concept that CD9 is a motility related protein. We suggest that the extracellular Fn-binding domain of the CD9 is important for cellular haptotactic motility.

1683

ErbB-3 RECEPTOR SIGNALING MEDIATES NEUREGULIN-INDUCED CELL MOTILITY AND MORPHOGENESIS A. Chausovsky, [1] M. Elbaum, [2] B. Geiger, [1] and A.D. Bershadsky.[1]. Departments of [1]Molecular Cell Biology and [2]Materials and Interfaces, The Weizmann Institute of Science, Rehovot 76100, Israel

Receptors of the ErbB family and their ligands play an important role in a variety of growth regulatory and morphogenetic processes. We have recently demonstrated that the ligand for ErbB-3/ErbB-4 receptors, neuregulin, can induce major morphogenetic changes in cultured epithelial cells, manifested by enhanced cell motility and conversion of epithelial islands into ring-like multicellular arrays with an internal lumen (Mol. Biol. Cell, 8: 10a). To define basic molecular requirements for the morphogenetic response to neuregulin, we transfected CHO cells (which form poor cell-cell adhesions and do not contain neuregulin receptors) with N-cadherin- and ErbB-3-encoding cDNA. These double transfected cells were shown to form ring-shaped arrays after 1-2 hours of stimulation with neuregulin. This process involves rapid activation of peripheral lamellipodial activity and changes in cell contractility that together lead to spreading of the colony and formation of a gap in its center. Ring formation can be suppressed by PI-3 kinase inhibition and by microtubule disruption or hyperstabilization. Migratory ability of single cells was also enhanced by neuregulin. Despite involvement of the ErbB-2 co-receptor in the neuregulin-ErbB-3-mediated signaling, direct activation of ErbB-2 (bypassing ErbB-3) neither enhanced single cell migration, nor induced colony to ring conversion. Thus, ErbB-3-mediated signaling leads to cytoskeletal reorganizations and activation of cell motility which, in cooperation with cell-cell adhesion, can create lumen-forming multicellular structures.

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SEPARATE SIGNALS ARE REQUIRED FOR ACTIVATION AND INACTIVATION OF THE p21-ACTIVATED PROTEIN KINASES (Paks) IN NEUTROPHILS. ((R.-Y. Huang, J. P. Lian, D. R. Robinson and J.A. Badwey)) Arthritis Unit, Massachusetts General Hospital and the Boston Biomedical Research Institute, Boston, MA 02114. (Spon. by J.A. Badwey)

Activation of Paks was compared in neutrophils stimulated with a wide variety of agonists. Paks were monitored by their ability to undergo renaturation and catalyze the phosphorylation of a peptide substrate that corresponds to amino acid residues 297-331 of the 47 kDa subunit of the NADPH-oxidase complex fixed within a gel. Neutrophils stimulated with sulfatide, a ligand for the L-selectin receptor, or the chemoattractants fMet-Leu-Phe (fMLP), platelet activating factor, leukotriene B_4, interleukin-8 or the chemokine RANTES exhibited a rapid and transient activation of the 63 and 69 kDa Paks. These kinases exhibited maximal activation with each of these agonists within 15 sec followed by significant inactivation at 3 min. In contrast, neutrophils treated with the chemoattractant C5a exhibited a prolonged activation (> 15 min) of these Paks even though the receptor for this ligand activates the same population of complex G-proteins as the fMLP receptor. Addition of fMLP to neutrophils already stimulated with C5a resulted in the inactivation of the 63 and 69 kDa Paks. While all of the agonists listed above triggered quantitatively similar activation of the 63 and 69 kDa Paks, fMLP was far superior to the other stimuli in triggering activation of the c-jun N-terminal kinase (JNK) and the p38-mitogen activated protein kinase (p38-MAPK). These data indicate that separate signals are required for activation and inactivation of Paks, and, in contrast to other cell types, activated Pak alone is not sufficient to trigger activation of JNK or p38-MAPK in neutrophils. These results are also consistent with G-protein coupled receptors initiating certain signals independent of those transmitted by their α and $\beta\gamma$ subunits. Supported by NIH grants DK50015 and AR43518.

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THE NEURAL-SPECIFIC RAC1B GTP-ASE SELECTIVELY ENHANCES NEURITOGENESIS. ((C. Albertinazzi, D. Gilardelli, S. Paris, A. Di Cesare, and I. de Curtis)) DIBIT - S.Raffaele Scientific Inst. 20132 Milano, Italy

Rho family GTPases have been implicated in cytoskeletal reorganization during neuritogenesis. We have recently identified a new gene of this family, Rac1B, specifically expressed in the chicken developing nervous system. The distinctive regulation of Rac1B expression suggests a role during neural development. In primary neurons, overexpression of Rac1B induces an increased number of neurites, and dramatically enhances neurite branching, while overexpression of the highly related and ubiquitous Rac1A GTPase does not affect neuronal morphology. Furthermore, expression of either an inactive or a constitutively active form of Rac1B strikingly inhibits neuritogenesis. The specificity of Rac1B action observed in neurons is not observed in fibroblasts, indicating the existence of a cell type-dependent specificity of Rac1B action. Molecular dissection of Rac1B function by analysis of the effects of chimeric Rac1A/Rac1B proteins shows that the carboxyterminal portion of Rac1B is essential to induce increased neuritogenesis and neurite branching, uncovering new mechanisms underlying the functional specificity of distinct Rho family GTPases. We have used affinity chromatograpy with GST-Rac proteins and embryonic brain extracts, to identify neural regulators and/or effectors implicated in Rac action during neuritogenesis. Electrophoretic analysis has allowed the purification of polypeptides which specifically interact with the active, GTP-bound Rac proteins. We are currently identifying the isolated proteins to study their function in Rac-mediated neuritogenesis. (Supported by the grant N.1028 from Telethon-Italy to IdC).

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DUAL ROLE FOR THE GTPASE, RAC IN CELL MIGRATION. ((Jie Leng, Archana Reddy, Richard Klemke, & David A. Cheresh)) Departments of Immunology & Vascular Biology. The Scripps Research Institute, La Jolla CA 92037

Cell motility depends on actin polymerization and depolymerization. Rac, a small GTPase impacts actin organization and the formation of lamellipodia and membrane ruffles, events important for cell migration. Evidence is provided that Rac potentiates cell migration on extracellular matrix proteins by two mechanisms. Not only does Rac promote membrane ruffles, but when expressed in cells in an active form it enhances, by 1000-fold, the ability of EGF to stimulate cell migration. We demonstrate that Rac is able to synergize with Raf kinase leading to increased MAP kinase activity. This, in turn, enhances the activation of myosin light chain kinase, an important regulator of actin-myosin motor function in motile cells. These results demonstrate that Rac GTPase activity regulates cell migration by promoting membrane ruffling and by influencing the MAP kinase pathway.

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Cell motility depends on actin polymerization and depolymerization. Rac, a small GTPase impacts the formation of lamellipodia and membrane ruffles. Evidence is provided that Rac potentiates cell migration on extracellular matrix proteins by two mechanisms. Not only does Rac promote membrane ruffles, but when expressed in cells in an active form it enhances, by 1000-fold, the ability of EGF to stimulate cell migration. We demonstrate that Rac is able to cooperate with Raf kinase leading to increased MAP kinase activity. This, in turn, enhances the activation of myosin light chain kinase, an important regulator of actin-myosin motor function in motile cells. These results indicate that Rac GTPase activity regulates cell migration by promoting membrane ruffling and by influencing the MAP kinase pathway. (Supported by grants CA50286, CA45728 and HL54444 (NIH) and DAMD17-96-1-6104 (U.S. Army Medical Research and Material Command)).